"If one attempts to attain an adequate understanding of the relation of an organism to its environment he addresses himself to a problem of such enormous complexity that he must, unfortunately, be reconciled from the outset to obtaining an incomplete answer. The task, which all scientists face, of isolation and simplification of problems is present in particularly acute form to the student of ecologically relevant physiology and behavior. He cannot reduce his problem until only a single variable remains; he cannot restrict his data to a single level of biological integration, nor, as is often the case in most other biological disciplines, even to several adjacent levels. Furthermore, he cannot limit his data gathering to techniques of any one specialty. More than most students, he must recognize that biology is a continuum. Whatever his techniques and methods, he must be a naturalist."

BARTHOLOMEW, G.A. 1966. Cited in *Readings in Aquatic Ecology* (edited by FORD, R.F. & HAZEN, W.E.), W.B. Saunders, Philadelphia, 1972.

ACCUMULATION AND EFFECTS OF CADMIUM IN THE OYSTER *CRASSOSTREA COMMERCIALIS*

A thesis submitted for the degree of Doctor of Philosophy at the Australian National University, Canberra, October, 1976.

This thesis is my own work except where specifically acknowledged.

Trevor Ward

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ABSTRACT

The Sydney rock oyster, *Crassostrea commercialis*, was exposed to chronic and sub-lethal levels of cadmium chloride in flowing sea water under laboratory conditions. The rate of accumulation of cadmium by oysters in the experimental system was high. From 10 µg/l cadmium in sea water the oysters accumulated 10 µg/g cadmium (dry weight) in approximately ten days. This rate of accumulation continued until the 16th week when the experiment was terminated. The amount of cadmium accumulated by the oysters is not related linearly to ambient levels of cadmium.

After long term treatment (eight weeks or more) the gut, gill, mantle and kidney reach the highest concentrations of cadmium. The gonad and the muscle do not appear to be important storage sites for cadmium.

The gut is the major route of accumulation of cadmium in the oyster, although the gill absorbs some cadmium directly from the water. Cadmium can be supplied to the gut in mucus and in food. The blood amoebocytes may scavenge cadmium from the gut and transport the metal through the tissues in the blood.

The oyster can accumulate similar amounts of cadmium from either particulate or soluble sources of the metal.

The localization of heavy metals by electron microscopy shows that most metal in the gill is bound by either amoebocytes, or by cytosomes in the apical area of the epithelial cells. The distribution thus demonstrated may be as a result of the two types of uptake

(through the gut and direct from water). Long term treatment with cadmium (10 and 50 µg/l) depresses the maximum rate at which oysters remove *E. coli* from suspension. Since *E. coli* may be filtered from suspension in a manner similar to the normal feeding process, cadmium may adversely affect feeding performance in the natural environment.

Long term cadmium treatment also has several effects on intermediary metabolism in the oyster gill, and causes some membrane "leakiness" or malfunction of active transport. However, energy levels within the gill are maintained by alternative pathways and biochemical indicators (ATP/ADP ratio, adenylate energy charge and adenylate pool size) show only marginal fluctuations even after long term treatment of the oyster with 50 µg/l cadmium.

The well known role of oyster amoebocytes in digestion and defence is extended to include the detoxification of soluble and particulate cadmium. The metal-binding characteristics of the amoebocytes may allow oysters to accumulate high concentrations of cadmium and show few resulting ill-effects.

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CHAPTER 1

INTRODUCTION

Cadmium is a trace metal found naturally at only very low concentrations. The toxicological literature often refers to cadmium as a "heavy metal", together with a number of other metals (mercury, lead, copper, zinc, etc.). "Heavy metal" is an imprecise term (Task Group on Metal Accumulation, 1973) and I consider it to be a non-systematic name, of limited chemical significance, for a poorly defined group of metals. The widespread use of the term in the literature suggests a mass acceptance by the scientific community. I therefore accept the term, but use it in this thesis with discretion.

At present, cadmium, together with two other common metals, mercury and lead, is not considered to be a biological requirement for living organisms (Vallee and Ulmer, 1972). As biochemical techniques advance it is conceivable that all three metals may, in the future, be found to play some natural role in metabolism.

The toxicity of mercury, lead and cadmium is a matter of common public concern. They are among the most common, highly toxic metals in the biosphere. Other metals may surpass their toxicity, but the ubiquitous distribution of mercury, lead and cadmium creates the potential for effects of a global nature.

Despite the widespread natural occurrence of many metals, and their recognized toxicity, it is only recently that very low levels have been noted as possible sources of danger to human health. Indeed, this threat to man has stimulated the flow of interest and funds to the

study of many potentially hazardous substances. As examples of the real hazard that heavy metal pollution represents, Minamata Disease and Itai itai were first detected in Japanese people. Minamata Disease is caused by the consumption of fish and shellfish containing high levels of mercury, while Itai itai was detected in people consuming rice from areas irrigated with cadmium contaminated water (Waldichuck, 1974; Friberg, et al., 1974). It is a sad, but necessary, comment on man and his society that it is only as a result of the Minamata Bay tragedy that significant research has begun into the effects and environmental behaviour of mercury. Similarly, Itai itai has stimulated much interest in the effects of cadmium in man, and its behaviour in ecosystems.

This thesis reports the results of an investigation into one aspect of pollution by heavy metals not often considered; that is, the long term effects of low concentrations of cadmium.

The potential for ecological damage by low levels of pollutants is great, since changes, such as alteration of population structures, behaviour patterns and reproduction may occur very slowly, and pass unnoticed. Biological resources remain uncatalogued, and their gradual demise as a result of the activities of man may be easily masked by the time scale involved.

The approach most often adopted to assess long term effects is the study of individual pollutants or processes, in an attempt to predict their effects. Use, production or disposal of the substance may then be limited accordingly. Laborious though this approach may be, there seems to be few feasible alternatives.

I have not attempted to review the literature in this

chapter, since a brief review of the relevant literature is included in the introductions to each of the four experimental chapters, and a general review would duplicate much information. As an alternative, and by way of introduction, I will examine some broad aspects of the presence of cadmium in the biosphere, and the significance of its distribution.

1.1 THE CHEMISTRY OF CADMIUM

Cadmium is found in group IIB of the periodic table, and is located close to zinc and mercury, with which it shares some chemical similarities. They are transition elements.

The toxicity of zinc to aquatic animals is generally considered to be lower than that of mercury and cadmium (Shuster and Pringle, 1969; Connor, 1972; Ahsanullah, 1976), but it is not clear, from chemical data alone, why this should be so (Pringle, et al., 1968). Mercury is higher than cadmium and zinc in electro-negativity (the tendency to acquire electrons in interactions), in the stability products of the sulphides, and in order of stability of the chelates (Pringle, et al., 1968; Perrin, 1975). However, both mercury and cadmium have much larger ionic radii than zinc (1.44, 1.41 and 1.25 Å respectively), which may be of some significance in toxicity. The toxicity of mercury is largely related to the speciation of mercury into molecular and atomic forms, and the biological transformation of Hg^{2+} to methylmercury. However, cadmium and zinc are not methylated in aqueous systems (Wood, 1975). The difference between the toxicity of cadmium and zinc may be largely governed by such factors as solubility in water and lipids, chemical speciation, interactions with

environmental factors, and environmental cycling.

1.2 BACKGROUND LEVELS OF CADMIUM

The natural occurrence of cadmium is widespread. Trace amounts are contained in many ore bodies and soils. Undoubtedly, geochemical processes will have contributed much cadmium to rivers and oceans from various metalliferous watersheds. Cadmium is extracted from ore bodies either for its own sake, or is carried along as a contaminant in other metal concentrates, and may remain as a residue in finished metal products. Cadmium may be released into the environment as a pollutant from both sources. The multitude of technological, industrial, agricultural and urban processes of a modern society contribute considerable quantities of cadmium to the environment, both aquatic and terrestrial. Preston (1973) considers that the contribution of cadmium to the oceans is not a global problem, since 10^4 years will be required to raise the level of cadmium in the ocean by 50% (on 1973 cadmium estimates). The more likely problem, according to Preston, is a local or regional pollution problem caused by cadmium. He presents data to support that theory by demonstrating that shallow inshore waters contain higher levels of cadmium than offshore waters. That situation may not be unique to cadmium. Evidence from studies on oysters (Ratkowsky, et al., 1974), and the study of water levels of cadmium (Knauer and Martin, 1973) support the concept of localized pollution involving several metals (zinc, cadmium, copper and lead). The levels of zinc, cadmium and copper increase in oysters which are closer to centres of urbanization and industrialization (Ratkowsky, et al., 1974). In a transect of the surface ocean waters between

Hawaii and Monterey Bay, California, Knauer and Martin (1973) could detect cadmium at only the two stations closest to the land masses.

Industrial processes likely to release cadmium to the environment include electroplating, battery manufacture, pigment manufacture (for paints, dyes etc), plastics production and electrical and electronics industries. The products of these industries may also release cadmium to the environment (for instance batteries, plastics and plated metal products). Urban wastes, such as road runoff and sewage, also contribute to the problem. It is therefore likely that cadmium is a widespread low level pollutant in many aquatic environments, but especially near areas of urban, industrial or agricultural activities, where high concentrations may exist in some local areas.

Background levels of cadmium in marine waters (those assumed to be contributed by natural geochemical processes) have been assessed only recently, and relatively few data are available. An average natural cadmium concentration reported for offshore marine waters is 0.02 µg/l (Knauer and Martin, 1973). Higher levels have been reported for the Bristol Channel, a near shore marine environment (up to 4.2 µg/l; Abdullah, Royle and Morris, 1972). Florence (1972) has reported a level of approximately 1 µg/l cadmium from surface waters of Jervis Bay, N.S.W., a relatively unpolluted marine embayment.

Background levels of cadmium from fresh water lakes and rivers in the U.S.A. may have a mean value of near 1 µg/l (Hem, 1972), although some samples from rivers in N.S.W. suggest that the mean level there may be lower (Doolan and Smythe, 1973).

The variability of levels of cadmium in natural systems reported in the literature is probably due to real variation in the systems. However, early studies of cadmium levels may have suffered from poor analytical techniques, and it is only the more recent data which is reliable (Friberg, Piscator, Nordberg and Kjellstrom, 1974).

1.3 POLLUTION

It is apparent that estuaries and inshore coastal waters receive fresh waters that may contain greater concentrations of cadmium than the oceans. Also, estuaries are commonly used as receiving waters and dumping grounds for industrial wastes and effluents, and urban wastes such as sewage, drainage and domestic refuse. Reclamation of intertidal areas is also a common practice in estuaries and sheltered bays. These actions are capable of adding, either continuously or in pulses, significant quantities of cadmium to the estuarine, and ultimately the marine, environments. The reduction of the natural intertidal areas of estuaries may reduce the ability of the system to detoxify and withstand the effects of pollutants.

The culture of the Sydney rock oyster (*Crassostrea commercialis*) is a multi-million dollar industry (the annual crop has been estimated by Mackay, et al., 1975, to be near $A. 10 million). The oysters are cultured on intertidal sticks and trays in most New South Wales estuaries. It is likely that the industry could be adversely affected by estuarine contamination with metals. Overseas experiences with oysters have shown that this is likely (Galtsoff, 1964) and locally, some problems have already arisen. Oysters from Ralphs Bay, Tasmania, have accumulated metals, particularly zinc from a

polluted estuary, to a level which is emetic in human consumers (Thrower and Eustace, 1973).

Williams and David (1973) report that superphosphate manufactured in New South Wales contains between 38 and 48 µg/g of cadmium, and that the metal is in a fully soluble form. Continued application of phosphatic fertilizers could conceivably raise the concentrations of cadmium in waterways near agricultural land.

Horvath, Harriss and Mattran (1972) have concluded that the Florida Everglades swamp lands are being polluted with heavy metals (including cadmium) from nearby agricultural lands, and state: "The chronic effects of cobalt and cadmium are unknown and the ecological significance of the enrichment of these metals in the Everglades estuaries cannot be established at present" (Horvath, Harris and Mattran, 1972).

Estuaries may, in the future, be the sites of much more intensive aquaculture than exists at present, perhaps for a range of animals and plants. It would seem highly appropriate to consider the sub-lethal and chronic effects of pollutants on estuarine organisms, in an attempt to assess levels which may be safely accommodated without an undue reduction in biological resources, and without prejudicing the aquaculture potential of estuarine and marine waters.

1.4 SOME DEFINITIONS

In this section I will define some terms used extensively throughout the thesis with, where appropriate, the original source.

Lethal: directly causing death.

Acute: lethal, directly causing death after a relatively short period of time (related to the normal life span).

Chronic: lethal, directly causing death after a relatively long period of time.

Sub-lethal: an effect not directly causing death.

The above four terms are liberal interpretations of definitions provided by Sprague (1969), and are consistent with dictionary definitions, except I define chronic to be purely lethal in accordance with Anderson (1975) but contrary to Sprague who considers chronic to be sub-lethal as well as lethal.

Short term: a time period which is brief compared to the normal life span of an organism.

Long term: a time period which is long compared to the normal life span of an organism.

It is difficult to set precise time limits within the definitions of short term and long term. I have adopted working definitions which may not be suitable to other molluscs. In my own experiments I regard the standard 96 hr test (Sprague, 1969) as short term, and eight weeks or longer as long term.

Tolerance: "the potential ability of animals to survive a certain environment for their normal life span" (from Anderson, 1975).

Resistance: "the ability of animals to survive in an environment which will eventually kill them" (from Anderson, 1975).

LC50: that concentration of toxicant that will kill 50% of a population within a specified time.

Incipient lethal level: that concentration of a toxicant which kills 50% of a population over an indefinite time.

Units: I have attempted to use standard units throughout the thesis, and have adopted the following where a choice was available:

Concentrations - Weights 1 µg/g = 1 mg/kg = 1 part in 10^6 parts.

1 µg/kg = 1 part in 10^9 parts.

Volumes 1 µg/ml = 1 part in 10^6 parts.

1 µg/l = 1 part in 10^9 parts.

1.5 *CRASSOSTREA COMMERCIALIS* ALIAS *SACCOSTREA CUCULLATA*

The Sydney rock oyster, besides being the largest single aquaculture industry in N.S.W., is also an important recreational resource, since it survives naturally and is eagerly sought in the intertidal zone of many estuaries and bays. This oyster is one of the few sessile, widely distributed intertidal molluscs on the east coast of Australia. It is a logical selection for a study of cadmium toxicity because it is commercially important, readily available, and studied in some detail in the past. Since the technology for collecting, transporting and culturing of spat are well known, any field trials suggesting themselves as a result of the study could be readily carried out. The laboratory rearing of young oysters has also been well studied (Galtsoff, 1964) and studies are continuing in attempts to assess accurately the natural food supply for adult oysters.

Although a convenient animal for study, the oyster may not be truly representative of estuarine molluscs in its response to high levels of cadmium. The high levels of cadmium, zinc and copper reported from oysters (for example Thrower and Eustace, 1973) could indicate a strong innate tolerance to high water levels of metals. If this is true, the extrapolation of results to other molluscs may not be appropriate.

Crassostrea commercialis (I and R) has been placed in a new genus, *Saccostrea*, by Stenzel (1971) on the basis of shell characters, and named *Saccostrea cucullata*. In a recent review Longwell (1976) discusses evidence from the crossing of several *Crassostrea* species which indicates that although a cross could not be obtained between *C. commercialis* and *C. virginica*, *C. irealei*, which may simply be a variant of *C. commercialis*, did display a cross with *C. virginica*. The situation is by no means clear. I have continued to use *C. commercialis* throughout the thesis, first because of the continued general acceptance of the name in Australia, and second, because of the confused state of the oyster taxonomy and systematics, it may be prudent to retain the most familiar recent name, in anticipation of a definitive study. The physiological differences between species of *Crassostrea* are unknown, and for practical purposes I have chosen to ignore any that may exist.

1.6 ACCUMULATION OF CADMIUM

1.6.1 VERTEBRATES

Cadmium derived from food, water and inhaled air can be readily absorbed, particularly by the lung, into the human body. The

subsequent transfer and storage is thought to be mediated by metallothionein (a metal-containing protein), resulting in the accumulation by the liver and kidneys of much of the absorbed cadmium (Friberg, et al., 1974).

In aquatic vertebrates, Olafson and Thompson (1974) have described cadmium binding proteins from the livers of the Atlantic grey seal *Halichoerus grypus*, and the Pacific fur seal *Callorhinus ursinus*. Olafson and Thompson (1974) report that the above proteins, together with a cadmium binding protein from a marine teleost, *Sebastodes caurinus*, have a molecular weight similar to that of metallothioneins from terrestrial vertebrates, and suggest they may function in a similar manner.

Other reports have indicated that aquatic vertebrates can accumulate cadmium (for example see Lovett, et al., 1972; Florence, 1972; Jaakkola et al., 1972).

1.6.2 INVERTEBRATES

The accumulation of cadmium by molluscs and crustaceans is well known (for example see Shuster and Pringle, 1969; Peden, Crothers, Waterfall and Beasley, 1973). However, most studies have been surveys of metal levels in the invertebrates, examining the whole organism or the edible flesh, and occasionally with the subsidiary aim of correlating levels in flesh with levels in water. Few studies have examined in detail the numerous ecological parameters associated with metal accumulation by the invertebrates; such factors as transfer through trophic compartments, efficiency and route of absorption, alteration of reproductive behaviour or success, genetic adaptation to

polluted environments, and others, remain to be described for most invertebrates.

1.7 THE TESTING OF TOXICITY

The effects of metals on aquatic animals have been investigated in numerous studies (partially reviewed by Bryan, 1971). The types of tests conducted fall into three main categories, acute, chronic and sub-lethal.

Assessment of acute effects usually involves an investigation of the 96 hr LC50 (concentration of toxicant which kills 50% of a population in 96 hr) and the incipient lethal level (the concentration of toxicant which kills 50% of a population over an indefinite time). From data on LC50's application factors can be used to predict "safe levels" for particular species. The extrapolation from acute levels ignores influences of environmental factors which may become relatively much more significant at chronic levels of pollutants. Further, other pollutants, or previous exposure to the same pollutant, and effects on other life stages can all be ignored using an extrapolation from acute effects to predict the overall toxicity of a pollutant.

Acute studies, whilst certainly having great value in toxicity testing, provide very limited data and should be carefully interpreted in the light of potential fluctuation of environmental variables. Also, the 96 hr test may only be applicable to organisms with a normal life span much greater than four days, since other animals may experience changes in life stage during the test.

The study of effects of metals at a sub-lethal level is

considerably more complex, which may explain the small number of investigations which have been reported in the literature. In many ways the sub-lethal effects may be as crucial to the long term survival of a population, and perhaps an ecosystem, as are the acute effects. The slow elimination of non-commercial species may well pass unnoticed until an irreversible stage is reached, and where, if environments have been sufficiently altered, effective recolonization is prevented. The sub-lethal effects which may gradually result in the demise of populations include subtle changes in reproduction, behaviour patterns, and physiological systems. The view that sub-lethal effects can be as detrimental to populations as acute effects is widely held in the literature (see, for example, Brown and Ahsanullah, 1971; Waldichuck, 1973).

The sub-lethal effects which are irreversible could well be considered to be the most critical for population success. A reversible effect could confer but a temporary disadvantage, from which a population may recover with no adverse effects. However, currently, it seems that even a temporary setback for a benthic community or population may have pronounced long term effects. Once established, benthic marine organisms may tend to alter the habitat for their own benefit, to the detriment of any competitors which may have previously dominated an area (Gray, 1974, 1976). Any temporary advantage could, in this way, be converted into a long term benefit by a species resistant to a particular toxicant. The complex spatial and temporal distribution patterns of benthic organisms leaves little room for confidently predicting the ecological effects of sub-lethal levels of toxicants in populations.

An extensive review by Waldichuck (1973) discusses in much greater detail the factors affecting acute and chronic toxicity testing, and presents a strong case for the careful monitoring of the effects of man on the marine biological resources.

In a detailed review of toxicity testing, data analysis and interpretations, and the prediction of "safe" levels, Sprague (1969, 1970, 1971) provides the basis for a standard approach to toxicity tests. Sprague recommends the adoption of standard techniques and approaches in mammalian physiology and biochemistry to deal with the assessment of effects of individual pollutants on aquatic organisms. There are many areas where such techniques could profitably be employed. In this study I have attempted to use a variety of techniques, selected not necessarily from mammalian biology, but rather from the biochemistry and cell physiology literature, with particular emphasis on reliability. As a result, few original techniques have been developed specifically for this study.

1.8 SYNERGISM, ANTAGONISM AND MODIFYING FACTORS IN TOXICITY

The concept of synergism and antagonism between pollutants in a mixture of pollutants is discussed by Sprague (1970). Few studies have examined the interaction of cadmium with other metals in their effects on aquatic invertebrates (Thorp and Lake, 1974). However, from experience with other metals some form of interaction could be expected (Gray, 1974; Waldichuck, 1973). Thorp and Lake (1974) found that zinc and cadmium are approximately additive in their effects on an aquatic crustacean, which perhaps indicates a similar mechanism

of toxic action.

All environmental variables are potential modifying agents, that is, capable of modifying the response of an organism to a toxicant (often observed in the literature as a change in the LC50). Such responses to environmental variables should be assessed as one part of the process of extrapolating laboratory data to field situations. The relation of water hardness (calcium and magnesium) to toxicity of metals to freshwater fish is well documented (Sprague, 1970). The other agents discussed by Sprague are temperature, dissolved oxygen, pH, and the CO_2-bicarbonate system (also in Brown, 1968). Probably the most important modifying factors for the toxicity of metals to marine invertebrates are temperature and salinity (Jones, 1973; Olsen and Harrel, 1973; Hutcheson, 1974; O'Hara, 1973) together with period of exposure for those animals that are intertidal.

Calcium levels, of great significance in fresh water, may not be as critical in marine or estuarine situations where the mean calcium concentration is approximately 300 mg/l, an order of magnitude higher than that in fresh water systems. Cloud (1965) reports that most coastal marine waters are supersaturated with calcium carbonate. Gardiner (1974a) cites unpublished work of Brown and Shurben (1973) which shows that the toxicity of cadmium is independent of water hardness.

In this study I have not attempted, for reasons of insufficient time and facilities, to assess the effects of other metal ions on the parameters being studied, nor have I investigated the effects of such environmental variables as temperature, salinity,

dissolved oxygen, feeding regimes, pH etc. on either accumulation or effects of cadmium. The study suffers as a result.

1.9 DESCRIPTION OF THE THESIS

Before commencing a study of the chronic and sub-lethal effects of cadmium on the oyster, it is necessary to delineate the areas of accumulation. Organs where cadmium accumulates may display the first, and perhaps the most severe effects, although such a tacit assumption is not well justified. Nonetheless, a knowledge of the sites of accumulation assists in the selection of organs for testing. The first experimental chapter (Chapter 4) describes the result of a single long term accumulation experiment designed to assess quantitatively the sites of accumulation, and gather some peripheral information on toxicity, weight loss, and possible relocation of cadmium in the body with increasing length of exposure.

Chapter 2 describes the experimental design, some underlying assumptions and limitations of the flowing sea water system developed to expose the oysters to low levels of cadmium.

Chapter 3 is essentially a brief, non-technical assessment of the biological significance of cadmium in sea water, and contains some theoretical concepts relating to the experimental approach I have adopted in this study.

In Chapter 5 the accumulation of cadmium is approached from two points of view by a consideration of the sub-organ and the sub-cellular levels. It complements the work in Chapter 4, and aids in its interpretation.

Chapter 6 is devoted to the study of some effects of cadmium on gill function, particularly feeding, and Chapter 7 follows with an examination of intermediary metabolism in the gill, studying effects of cadmium on enzyme systems and energy production.

The final chapter, Chapter 8, reviews the major findings of the study, and presents some speculation about the likely physiological, biochemical and ecological implications.

CHAPTER 2

THE FLOWING SEA WATER SYSTEM

Acute toxicity tests are often performed in static systems, that is, where the water is changed manually on a regular basis, or not at all if the test is very brief. Although this method is applicable to chronic and sub-lethal tests as well, it is laborious and time consuming, and allows the build-up of excretion products and the depletion of toxicant. The concentration of toxicant being tested may fluctuate markedly if the ratio of volume of test solution to biomass of organism being tested is low, complicating the interpretation of toxicity.

At very low toxicant concentrations the loss of toxicant by animal absorption could become a major modifying factor in the expression of detrimental effects. The alternative to the static test is a flowing system, where the water is continuously replaced, and toxicant solution is continuously supplied to maintain concentrations, simulating a constant environmental level. If the rates of flow are great enough the materials excreted by the organisms are removed, and the reduction of toxicant levels by the animals can be ignored. An added advantage, in the case of oysters, is that the incoming water may be used to supply natural food organisms.

In both types of test, a careful assessment of the culture conditions is needed before the beginning of tests to determine optimum, or tolerable, conditions for the organism being tested. I conducted several static trials with oysters, at the density used for

the experiment reported in Chapter 4, and after four weeks, mortalities, general appearance and response all appeared satisfactory, provided the water was changed weekly. Initially, a feeding supplement was provided (finely pulverized corn flour), but this was soon abandoned from the preliminary trials because of a rapid subsequent reduction in water quality in the tanks (growths of algae, etc.). The addition of the food supplement appeared to have little beneficial effect on the oysters' condition.

To maintain levels of toxicant a slow running system was adopted as a vehicle for long term exposure of the oysters to cadmium. The sea water was renewed once a week in the holding tank, and I assumed that this would provide some natural food for the oysters. Although there was some weight loss, control oysters survived well and appeared normal throughout all the experiments reported in this thesis (see Chapter 4).

The experimental tanks used throughout are all glass 70 x 29.5 x 22 cm aquaria, with a perforated perspex sheet suspended 10 cm from the bottom and an overflow pipe at 4.5 cm from the top of the tank. The effective volume is 38 litres. The false bottom allows the tanks to be easily cleaned of faeces and pseudofaeces. Toxicant and sea water are fed into the tanks at one end, and kept well mixed by two air stones per tank. The air was supplied initially from a reticulated air supply (while the system was in Canberra) but later a diaphragm type air pump was used.

The sea water was gathered weekly from Batemans Bay or Durras (coastal locations near Canberra) and transported by road in all polyethylene containers to Canberra. All pumps, tubing and tanks

were plastic or glass. A 1,800 litre covered tank was kept outside the building, and water was pumped into the constant temperature laboratory as required. I attempted to maintain the salinity near that of full sea water for all experiments, unless otherwise specifically noted. The conductivity and pH of the sea water used is shown in Table 2.1.

TABLE 2.1

	mean pH ± s.e.m.	Conductivity (mhos/cm) mean ± s.e.m.
5/3/74 to 17/7/74 (Canberra)	7.8 ± 0.07	1.09 ± 0.04 (26^{o}/oo)
March, 1975 to March, 1976 (Cronulla)	n.d.	1.38 ± 0.02 (35^{o}/oo)

The conductivity of the sea water was checked at weekly intervals to ensure near marine salinities, and on one occasion (24th April, 1974) the water was artificially raised to a higher salinity using commercially available coarse sea salt. Conductivity was used to achieve approximate salinity. The conductivity of an artificially prepared sea water (34^{o}/oo) and the conductivity of surface coastal waters (approximately three miles offshore) were in very good agreement. The conductivity of the natural sea water (at 20^{o}C) was 1.31 mhos/cm, and was linearly related to % sea water down to 25% sea water (diluted with distilled water) (0.37 mhos/cm). No attempt was made to simulate tidal fluctuations in the experimental tanks, and it is not known what effect continuous exposure to sea water has on previously intertidal oysters. However, beds of *C. virginica* have been found as deep as 40 m, containing oysters in apparently viable and healthy states (Merill and Boss, 1966). Also, Shaw (1963) and Galtsoff (1964) describe the successful growing of oysters on rafts,

presumably continually immersed in sea water.

Oysters maintained for long periods under the controlled conditions ($20^{o}C \pm 2^{o}C$, 12/12 light regime) appeared (on a highly subjective basis) to feed primarily at night, after several weeks acclimation. The observation suggests that the oysters rapidly adjust to laboratory conditions. The ecological implications of night time feeding by totally immersed oysters are interesting (if this occurs in the field). It is possibly an adaptation to avoid fish predators (those that use visual cues) and also may exploit the diurnal migration of estuarine and marine plankton populations towards the surface of water bodies at night.

I have assumed that continuous exposure to water of fully marine salinities (approximately 34^{o}/oo) is not detrimental to oysters. This is supported in part by results of Davis (1958) who shows that 26 to 27^{o}/oo is the optimum salinity (higher salinities were not tested) for reproduction and early growth of *C. virginica*.

Water samples were taken weekly from all experimental tanks, and cadmium analyzed after storage in acid soaked (5% nitric) polyethylene bottles at pH < 3 and $4^{o}C$. The method of analysis of cadmium in sea water was adapted from Yeager, Cholak and Meiners (1973) (for complete details see Appendix 3).

In November, 1974 the complete flowing system was moved to the laboratories of CSIRO, Division of Fisheries and Oceanography, Cronulla, N.S.W. After a settling period for the system the only substantial difference between the two locations was that water samples were taken only at two weekly intervals, when the system

received regular maintenance (cleaning, replacement of tubing, etc.). The salinity of the water in the Cronulla laboratory system was also tested every second week, and was always close to $34^{o}/oo$. Water temperatures fluctuated between $17^{o}C$ and $21^{o}C$. The sea water used was buffered against seasonal changes in water temperatures by its residence time in the temperature controlled laboratory.

The sea water was taken from two sources simultaneously, a very large cement swimming pool adjacent to the laboratory, and the filtered reticulated supply. The two waters were mixed in a plastic head tank (shown in Figure 2.1, the diagrammatic representation of the system at Cronulla). The two sources of water ensured a "failsafe" mechanism for the sea water supply, since they were independent of each other. The cadmium supply system was not "failsafe", and after the three occasions that the system was disrupted, the experiment was abandoned. On one of these occasions (a power failure for several days during January, 1975) nearly four months were lost when an experiment had to be abandoned.

Because of practical constraints, the flow rate of sea water had to be kept to a minimum, especially in Canberra. Following the successful survival of oysters for four weeks in a static system, it seemed that a low sea water flow might be acceptable. The loss of toxicant by absorption and adsorption seemed to be the only major obstacle. The latter was minimized by careful ageing of the tanks for at least two weeks prior to adding the oysters. The calculated concentrations of cadmium in the tanks agreed well with the levels observed by analysis, so, in retrospect, that approach was justifiable.

The flow rate adopted was 1 l/hr tank, with a suitable flow of toxicant solution (all less than 4 ml/hr/tank). Oysters used as controls in the flowing system at Cronulla flourished, adding new shell, considerable "fatness", and appeared in very good health, for as long as six months. The control oysters from the flowing system in Canberra lost weight over the 16 week experimental period, (reported in Chapter 4) and although in a quite acceptable condition, had probably declined through insufficient food.

The flow rate chosen (1 l/hr) results in a 50% replacement of the water in the tanks in approximately 22 hr (Sprague, 1969). The minimum water:biomass ratio suggested by Sprague (1969) for fish is 1 l/g, changed daily. Assuming an average wet weight of 5 g/oyster, and a maximum of 60 oysters per tank, the minimum flow rate, as suggested by Sprague, would be approximately 12.5 l/hr. The calculations cited by Sprague (1969) are based on oxygen consumption requirements of fish, and are of dubious value for extrapolation to the requirements of oysters. A flow of 10 l/hr would have been desirable, but was beyond all practical possibilities. The natural food content of the water used in the flowing system at Cronulla was high through most of the year, and judging by the oysters' growth, provided a suitable laboratory environment.

The toxicant solution was prepared using cadmium chloride ($2\frac{1}{2}$ H_2O) (BDH, Analar) dissolved in filtered sea water. A 100 µg/ml stock solution was prepared and adjusted to pH 3, and a further stock of working solutions (in polyethylene containers) was prepared in filtered sea water as required, without adjusting the pH. The cadmium solution was pumped directly from the working solutions into the

experimental tanks (filtered sea water into the control tank). Experiments were always commenced by adding oysters to the tanks from similar tanks in which they had been acclimated to the laboratory conditions. In this manner the flow of toxicant was not interrupted and the tanks were aged in the appropriate concentration of cadmium prior to the addition of oysters.

The concentration of cadmium in the tanks, assessed weekly from 5/3/74 to 17/7/74 (Canberra) and fortnightly (Cronulla) is shown in Table 2.2. Cadmium levels from experiments that were discarded through equipment malfunction are not included.

Oysters used in all experiments were obtained through commercial sources from the Clyde River, Batemans Bay, N.S.W. The source was authenticated at each purchase. The oysters obtained in this manner are a mixture of stick and tray grown oysters, ranging in age from 2½ to 3½ years old. Before use all oysters were scrubbed with a nylon bristled wooden brush to remove mud and epiphytes. The acclimation period was usually four weeks, although this was shortened to two weeks in some cases.

The oysters used in the experiment described in Chapter 4 were approximately 8 cm in height (mean of 60 oysters). The mean wet weight of oysters used in all experiments was approximately 5 g.

The complete flowing system is outlined diagrammatically in Figure 2.1.

TABLE 2.2

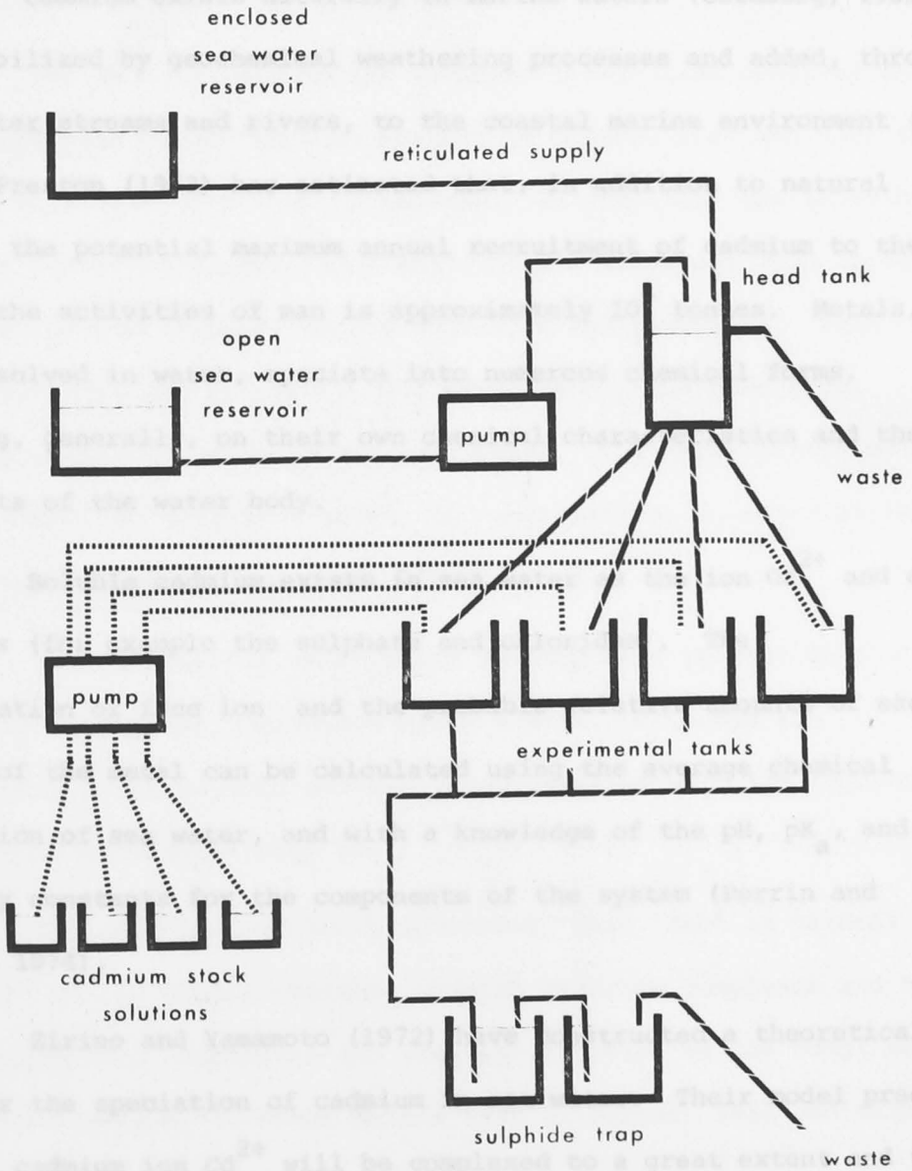
Concentration of cadmium in water samples from the flowing system (µg/l).

Nominal cadmium level	0	10	25	50	100	150
Number of determinations	14	36	12*	38	12*	7*
Mean level of cadmium (µg/l) ± s.e.m.	b.l.s.	9.8±1.07	24.1±5.2	46.3±2.6	90.1±6.3	149.2±15.9

* After the move to Cronulla the 25 µg/l level was discontinued, the 150 µg/l was adjusted to 100 µg/l, and finally the 100 µg/l level was discontinued. Controls were all below the level of sensitivity of the assay system (1 µg/l).

FIGURE 2.1

THE FLOWING SEA WATER SYSTEM



CHAPTER 3

CADMIUM IN SEA WATER

3.1 SOLUBILITY AND SPECIATION

Cadmium exists naturally in marine waters (Goldberg, 1965). It is mobilized by geochemical weathering processes and added, through fresh water streams and rivers, to the coastal marine environment (Hem, 1972). Preston (1973) has estimated that, in addition to natural sources, the potential maximum annual recruitment of cadmium to the sea through the activities of man is approximately 10^4 tonnes. Metals, when dissolved in water, speciate into numerous chemical forms, depending, generally, on their own chemical characteristics and the components of the water body.

Soluble cadmium exists in sea water as the ion Cd^{2+} and as complexes (for example the sulphate and chlorides). The concentration of free ion and the probable relative amounts of each complex of the metal can be calculated using the average chemical composition of sea water, and with a knowledge of the pH, pK_a, and the stability constants for the components of the system (Perrin and Dempsey, 1974).

Zirino and Yamamoto (1972) have constructed a theoretical model for the speciation of cadmium in sea water. Their model predicts that the cadmium ion Cd^{2+} will be complexed to a great extent and that cadmium complexes with coordination numbers (metal - ligand interactions for any one metal atom) higher than two are probably of no significance. Also, their model predicts:

1. the free cadmium ion constitutes 2.5% of the total cadmium;
2. $CdCl_2^0$, $CdCl^+$, and $CdCl_3$ account for 95% of the total;
3. of the other possible complexes in sea water, only the carbonate and hydroxides exist (together, less than 1% at pH 8.0); and
4. the distribution of cadmium between the species is not affected by changes in the pH (in the range 7.0 to 9.0).

The model of Zirino and Yamamoto (1972) also predicts that the distribution of the various species of copper, lead and zinc in sea water is different from that of cadmium. In addition, the types of complexes formed by copper, lead and zinc are different from those formed by cadmium (copper, mainly hydroxide and carbonate; lead, mainly carbonate and chloride; and zinc, mainly the hydroxide, chloride, carbonate and sulphate complexes). The three latter metals exhibit strong pH dependent shifts in complexation, in contrast to cadmium which shows little or no effect of pH.

The theoretical model above is supported in part by the observations of Ernst, Allen and Mancy (1975). Although the experimental evidence does not correspond exactly with the theoretical model, it is consistent with it.

The available evidence indicates, then, that in natural sea water and, at low concentrations, cadmium chloride complexes and the free cadmium ions constitute the greatest fraction of the total cadmium (98.5%), in contrast to other metals such as copper, lead and zinc.

In fresh water a comparable situation does not exist. Gardiner (1974a) demonstrates that the great majority of the soluble cadmium in fresh water is uncomplexed free ion (Cd^{2+}). Up to

40% of the total cadmium may be complexed as hydroxide, chloride, carbonate or sulphate in filtered river water. The distribution of cadmium between those species above (including the free ion) is dependent upon pH. The presence of humic acid dramatically increases the fraction of complexed metal in fresh water (Gardiner, 1974a).

In addition to the existence of metals in a number of soluble species, both complexed and free, metals can be associated with insoluble components of natural waters, especially as colloids, precipitates and organically and inorganically adsorbed metal. Biological adsorption is also important, but this will be discussed separately.

Gardiner (1974b) demonstrates that humic acid (in suspension) has a strong affinity (stronger than silica, kaolinite, fish faecal material, and plant material) for the adsorption of cadmium from a fresh water system. However, Gardiner predicts that only at high concentrations of suspended solids (greater than 20 mg/l) will the concentration of soluble cadmium fall below 50% of the total cadmium. It seems then that, even in fresh water situations of high suspended solids load, most cadmium remains soluble. Although the pattern of speciation is different from that in sea water, cadmium is likely to remain mostly soluble in both sea water and fresh water systems. Gardiner (1974b) shows that when a soluble complexing agent (EDTA) competes, in a fresh water system, with an insoluble adsorbing surface (river mud) for cadmium ions, there can be strong reduction in the amount of metal adsorbed to the insoluble surface (an increase in the amount of soluble metal). If this principle can be applied to sea

water, it is apparent that the chloride complexes could effectively compete with insoluble adsorption sites (clay particles, humic acid, organic detritus) for cadmium, and increase the amount of soluble metal.

The presence of materials adsorbing metals is suggested by Gardiner (1974b) as providing a buffer or store of metal for the soluble phase. Since adsorption and desorption are rapid, removal by say, biological accumulation, of soluble ionic metal can cause a rapid release of adsorbed ions to the soluble fraction. This may mean that the metal adsorbed onto insoluble material in water may be as available, in toxicological terms, as the soluble metal. If both this assumption and that of the preceding paragraph (that complexation desorbs metal from insoluble sites) are correct it is apparent that cadmium in sea water will exist in the soluble form, independently of the insoluble solids load.

In fresh water, in the absence of other ligands, Shaw and Brown (1974) show that the toxicity of copper is related to the total soluble copper concentration (the free copper ion and copper carbonate). In a more common situation, where natural organic ligands are present, in fresh water, Brown, Shaw and Shurben (1974) show that the toxicity of copper is more directly attributable to the concentration of soluble copper than to that of the insoluble copper. As a result, the distribution of a metal between the various possible species can be assumed to have a significant influence on its toxicity to aquatic organisms (Gardiner, 1974a).

Aquatic animals feeding on particulate matter could be expected to ingest considerable amounts of metal if a metal existed

mostly adsorbed onto organic or inorganic particles in suspension. Similarly, those animals feeding by filtering large volumes of water may accumulate a large amount of metal directly from the water if the soluble ionic species of a metal is predominant. Unicellular aquatic organisms may be the most sensitive to dissolved ionic species because of their surface to volume ratio, and the nature of their feeding mechanisms. It seems likely, on the basis of the available evidence, that cadmium exists in sea water as soluble chloride complexes, with a small percentage as the ionic form, independent of pH and suspended organic and inorganic solids.

3.2 AGENTS MODIFYING SPECIATION

Environmental agents which influence the distribution of a metal among its possible species without necessarily altering the total concentration are modifying agents. Modifying agents can also alter toxicity. Calcium is a well known modifier of the toxicity of heavy metals to fish (Sprague, 1969). The effect of calcium may be chemical (to change the speciation of the metal), biochemical (to compete for uptake sites) or physiological (to protect against toxicity). The data of Brown, Shaw and Shurben (1974) suggest that the first possibility may be the most important for the toxicity of metals to trout in fresh water. Agents modifying metal speciation need not also modify toxicity.

Chlorinity may be the most important modifier of the speciation of cadmium in sea water and, if so, will be most active in estuarine situations, where salinities often fluctuate markedly. The effects of the possible modifiers temperature and dissolved oxygen are unknown. One further important modifier of speciation is biological

absorption, which, as with the other modifiers of speciation, may not necessarily change the total concentration of metal, but simply shift the equilibrium positions of the possible species.

Phytoplankton are known to accumulate strongly trace metals from sea water (Goldberg, 1965), and they may play a significant role in the reduction of the levels of soluble metals in the upper layers of the ocean (Spencer and Sachs, 1970). Knauer and Martin (1973) show that plankton absorption is responsible for the seasonal fluctuations in soluble cadmium levels in open ocean waters. Metals bound by plankton may be subsequently released to the environment at a different trophic level or, in shallow waters, may be incorporated into sediments.

The environmental factors controlling the growth and reproduction of phytoplankton and zooplankton may also influence the levels of soluble cadmium in sea water. Once absorbed, it is unlikely that the biological pool of cadmium would be able to directly contribute to the soluble pool of cadmium, although predation upon, or death of, plankton may indirectly make a contribution.

Although absorption of metals by plankton may not alter the total water concentration of metal in open ocean waters, in shallow coastal waters cadmium could be desorbed from sediments by blooms of plankton. Such desorbed metal would be returned to the water column in the insoluble phase. Helz, Huggett and Hill (1975) suggest possible mobilization of cadmium from sediments to explain an anomalous increase in cadmium concentrations as a river became more saline. The increase of cadmium levels towards the mouth of the river was not consistent with the pattern of the other metals examined, and was not

due to dilution with sea water of higher cadmium concentration. The anomalous increase could be explained by either the effect of chlorinity, or an increase in plankton populations.

Few experimental studies have attempted to assess both the particulate and the soluble fractions of any cadmium level in sea water. Florence (1972) reports variable levels of soluble and particulate cadmium from Jervis Bay, N.S.W., but Preston (1973) reports that 80% of cadmium from U.K. coastal waters is present as a soluble form.

In summary, available experimental evidence suggests that most cadmium in sea water exists in the soluble form and, from theoretical and limited experimental observations, that the soluble form may be mostly the chloride complexes, with a minor proportion of free ion. Also, the rapid desorption of cadmium from the insoluble phase may buffer the level of metal in the soluble fraction. It is therefore possible that the toxicity of cadmium is more closely correlated with the total (soluble and insoluble) cadmium level, in contrast with the situation of copper in fresh water. The factors controlling the level of the soluble species of cadmium in sea water are (in probable order of significance):

1. chlorinity, providing a soluble complexing factor;
2. biological absorption, reducing the soluble level; and
3. adsorption on to insolubles in suspension, providing a store of metal for the soluble fraction.

As a direct consequence of the effect of chlorinity on the distribution of cadmium in sea water, the fixation of cadmium into sediments is likely to be small, and the concentration factor between

sediments and sea water is likely to be lower for cadmium than for other metals.

3.3 COLLECTION AND PRESERVATION OF WATER SAMPLES

The collection and preservation of water samples for trace metal analysis has been the subject of several investigations. Gardiner (1974b) has reported the loss of cadmium to container walls (fresh water). Smith (1973) has shown that 15 metals (not including cadmium) require a pH of 2 or less to be maintained in solution. However, a definitive study of the adsorption of cadmium from water onto borosilicate glass and polyethylene has been reported by Struempler (1973). In that study the adsorption of cadmium onto borosilicate glass was linear at pH 6 (at day 20, 20% of the ions were adsorbed). Acidification of the water resulted in a linear desorption. At an initial pH of 2 no cadmium was lost to the surface of the glass or the polyethylene containers. Polypropylene containers gave high blanks and were not studied further by Struempler (1973).

It follows that, the appropriate collection technique should involve acidification of the water sample (either filtered or unfiltered) to pH 2. The preferred storage container is high density polyethylene, to avoid adsorption of metal and evaporation of solvent. Storage at $4^{o}C$ or below may assist in retarding the growth of moulds, etc. and is unlikely to influence metal levels.

3.4 ASSAY TECHNIQUES

Numerous techniques are now available for the assay of cadmium in sea water at the µg/l level. Neutron activation and anodic

stripping voltammetry are probably the most sensitive techniques, although the atomic absorption techniques (AAS) appear to be the most widely available and most popular. The AAS techniques require a preconcentration step, several of which are available (chelating resins, chemical flocculents, solvent extraction). I have chosen a solvent extraction method (Appendix 3) followed by flame AAS for the study reported in this thesis. The technique may also be suitable for the detection of sub-µg/l levels (near background) using flameless AAS, but I have not attempted to investigate that aspect. The technique employed, coupled to flame AAS, is suitable for assessment of cadmium levels in sea water of between 1 and 50 µg/l.

The levels of particulate cadmium (> 0.45 µm), in five spot tests of cadmium levels in the flowing sea water system (Chapter 2) were between 0 and 10% of the total cadmium.

CHAPTER 4

ACCUMULATION

4.1 INTRODUCTION

Numerous studies have shown that marine invertebrates accumulate metals from both unpolluted and polluted natural waters (e.g. Windom and Smith, 1972; Peden, Crothers, Waterfall and Beasley, 1973). Similarly, many studies have shown that marine invertebrates can accumulate metals from sea water under laboratory conditions (e.g. Pentreath, 1973; O'Hara, 1973; Eisler, Zaroogian and Hennekey, 1972).

Crassostrea commercialis is capable of accumulating cadmium from low concentrations. Mackay, Williams, Kacprzac, Collins and Auty (1975) have reported a mean level of cadmium in oysters, from relatively unpolluted N.S.W. waters, of 0.1 µg/g (wet weight). Nielsen and Nathan (1975) report a mean level of cadmium in the New Zealand rock oyster (*C. glomerata*) of 1.3 µg/g (wet weight). The level of cadmium in unpolluted sea water is approximately 0.1 µg/l (Florence, 1972; Goldberg, 1965).

Ratkowsky, Thrower, Eustace and Olley (1974) have observed levels of cadmium in oysters from polluted waters (Ralphs Bay, Tasmania) of up to 19.8 µg/g (wet weight). Although water levels were not reported, it is clear that the oysters had accumulated cadmium from their polluted environment. Overseas studies with oysters of the *Crassostrea* genus have also demonstrated the ability of these oysters to concentrate cadmium from their environment. Concentration factors (the level of cadmium in tissue/level of cadmium in water) of 1,000 to

100,000 have been reported (Brooks and Rumsby, 1965; Kopler and Mayer, 1973; Nielsen and Nathan, 1975).

Laboratory studies have examined more rigorously the ability of oysters to concentrate cadmium from their environment (Brooks and Rumsby, 1967; Shuster and Pringle, 1969; Eisler, Zaroogian and Henneky, 1972; and Zaroogian and Cheer, 1976).

There is no known biological requirement for cadmium by oysters, so the accumulation of cadmium is not in response to a requirement for the ion *per se*, or a requirement for cadmium as a substitute ion for some other cation, in metabolic processes. It is possible however, that cadmium is absorbed because of its chemical similarity to zinc, an element essential for many metabolic functions.

The accumulation of cadmium by the organs of the oyster has not been studied in detail. In some vertebrates it is clear that some organs function as accumulators of cadmium while others remain relatively free of cadmium (Friberg, Piscator, Nordberg and Kjellstrom, 1974). In an extensive study of the distribution of trace metals in molluscs (other than oysters) Segar, Collins and Riley (1971) have described the distribution of cadmium (amongst other metals) in the organs of several bivalves. The study showed that the shell is a minor accumulator of cadmium (consistent with the data of Brooks and Rumsby, 1965) and that, in *Pecten maximus* and *Modiolus modiolus*, the gut, and the gills and mantle, contained the highest concentrations of cadmium.

Fowler and Benayoun (1974) found that, in *Mytilus galloprovicialis*, the shell accumulated the least ^{109} cadmium in an experimental system. However, the shell had the highest concentration

of stable cadmium, indicating a lack of turnover of cadmium. Although of considerable ecological significance, the role of the shell as an accumulator of cadmium is not investigated in this thesis.

Pentreath (1973) has examined the organ distribution of ^{56}Zn, ^{54}Mn, ^{58}Co and ^{59}Fe in *Mytilus edulis*, Cunningham and Tripp (1975a) have similarly examined the organ distribution of ^{203}Hg and $CH_3{}^{203}Hg$ in *C. virginica*, and Brooks and Rumsby (1967) examined the organ distribution of cadmium (amongst other metals) in *Ostrea sinuata*. Brooks and Rumsby (1967) found that the gill, the visceral mass and the heart/kidney accumulated large amounts of cadmium after challenge with a massive dose of cadmium (50 mg/l).

4.1.1 INFLUENCE OF ENVIRONMENTAL FACTORS ON ACCUMULATION.

The accumulation of cadmium by bivalves is probably influenced by fluctuations in normal environmental variables, although this has not been specifically studied in oysters. The accumulation of cesium by a bivalve is known to be affected by changes in temperature and salinity (Wolfe and Coburn, 1970), and temperature and salinity affect the accumulation of cadmium, and its organ distribution, in the crab *Uca pugilator* (O'Hara, 1973). Cunningham and Tripp (1975b) have demonstrated an effect of temperature on the biological half time of mercury in *C. virginica*. Zaroogian and Cheer (1976) have attributed a variable rate of cadmium accumulation in *C. virginica* to the effects of temperature, although Fowler and Benayoun (1974) provide experimental evidence which indicates that the rate of cadmium accumulation in *Mytilus galloprovincialis* is not affected by temperature. It is evident that environmental variables such as temperature, salinity and feeding regimes may influence accumulation and depuration, and that

this aspect warrants further investigation.

4.1.2 EFFECTS OF CHRONIC LEVELS OF CADMIUM

Although many studies have described the accumulation of cadmium in marine invertebrates, most were not concerned with the effects of the body burden of the metal on the animal itself, but rather were concerned about possible human health hazards. Few studies have examined the chronic or sub-lethal effects of accumulated cadmium in any animal, least of all in marine invertebrates (Bryan, 1971).

Schuster and Pringle (1969) have described the effects of 0.1 and 0.2 mg/l cadmium exposure on the shell growth and general appearance of *C. virginica*, although the physiological effects on the animal were not described (apart from mortalities). No other studies have examined the chronic effects of cadmium on oysters.

Before turning to a study of the effects of the body burden of cadmium on the oyster it is necessary to first clearly delineate the areas of accumulation within the body, and their quantitative relation to each other. Implicit in this approach is the assumption that organs that accumulate cadmium will show effects of that accumulation. There is a considerable body of circumstantial literature, mainly from the vertebrates, to support this assumption (Friberg, et al., 1974; Winge, et al., 1974). However, it is possible that the effects of cadmium limiting the viability of an organism are not displayed at the site of greatest accumulation of metal in the body.

In a chronic cadmium challenge Nordberg (1972) reports that mice show accumulation of cadmium in the testes, the salivary glands, and the pancreas, although it is the kidney which is the "critical

organ" (the "critical organ" is the organ which first reaches a concentration of metal that causes undesirable cellular malfunctions; for a further discussion see Task Group on Metal Accumulation [1973]). Similarly, Nordberg (1972) discusses the relation between the critical organ and the organ concentration of metal, and dismisses any clear link between the two. After an acute dose of cadmium, the testes of mice showed obvious histological damage whilst, after a chronic dose, and despite higher accumulated levels of cadmium, they displayed no apparent damage. Taken at face value, it appears that there is no direct proof to support the assumption that the site of accumulation of cadmium is the site of effect, or, more correctly, the site of the viability limiting effect. Perhaps a clearer picture would emerge if functional, rather than structural, criteria were employed to assess effects. In any case, and despite the uncertainty about other animals, in mice and rats the critical organs (acute dose - testicle; chronic dose - kidney) do accumulate cadmium, although they do not necessarily accumulate the greatest amount of cadmium.

In summary, it seems that for some vertebrates, the critical organs may well be significant accumulators of cadmium, although the converse may not necessarily hold. The situation in oysters is completely unknown but, given the limitations described above and below, an initial investigation for effects of cadmium should be directed at organs that can be identified as accumulators of cadmium. Such a naive approach ignores a number of obvious possibilities such as effects on membranes, disruption of cell function at sites of absorption, effects in blood and other body fluids, etc., but further justification of the selection of any particular approach to the problem, given the paucity

of available information, borders on the trivial.

4.1.3 THE BIOLOGICAL BASIS FOR THE TOXICITY OF CADMIUM

Toxic effects of cadmium are observed once the rate or magnitude of the protective mechanism(s) has been exceeded. It is possible that, in some vertebrates, the protective mechanism, involving numerous organs, is the production and distribution of the protein, thionein (Friberg, et al., 1974).

There is little evidence for the existence of a metallothionein in invertebrates. Indeed Coombs (1974) reports the absence of any specific metal binding proteins when examining the distribution of zinc and copper in *Ostrea edulis*.

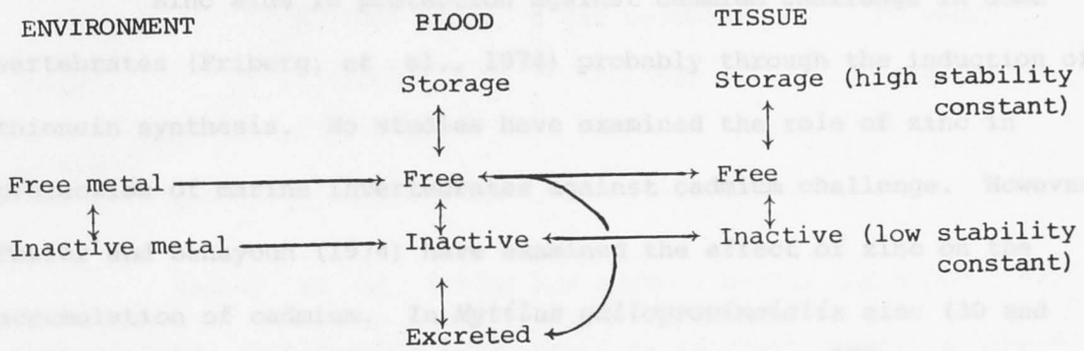
Ultimately, the toxicity of a constant environmental level of cadmium, once absorbed, depends on:

1. the nature of the site at which the metal is bound;
2. the location of that site in the body;
3. the intracellular or local concentration of metal;
4. the sensitivity of physiological and biochemical systems to inhibition by cadmium;
5. the ratio of metal ions to available high affinity binding sites; and,
6. the rate at which the metal can be excreted from the body.

The free ion (Cd^{2+}) is very "active" and unlikely to exist for long periods of time in biological fluids in such form. However, it may be the mean concentration of the free ion which is responsible for the toxic effects (Task Group on Metal Accumulation, 1973).

Many biologically significant molecules (proteins, amino acids, etc.) are capable of binding cadmium, with varying degrees of

effectiveness. It is probably those molecules with lower stability constants for cadmium binding (lower affinity for cadmium) that allow a small fraction of the total cadmium load to remain in the free ionic form. Such a concept is shown diagramatically below.



The stability constants for the binding of the metal to various biological compounds dictate the levels of free ion, which are probably closely correlated to the magnitude of the toxic effects of the metal. Some stability constants for cadmium and various molecules are listed by Vallee and Ulmer (1972).

The discussion above is a greatly simplified interpretation, and is unlikely to be capable of explaining completely either the distribution or the toxicity of cadmium in biological systems. The great range of potential ligands, with affinities ranging from negligible to so high as to be essentially irreversible, serve to mask the sites of effect, and diffuse the metal through numerous tissues. The affinity of a site for cadmium may have no relevance to the magnitude of the effect that cadmium has if it binds to a ligand, although it may well dictate the residence time of the ion at the site.

The ability of any organism to modulate the effects of

cadmium may also depend on such mechanisms as the maximum rates for synthesis of replacement proteins, natural turnover rate of structural components, reliance on substitute systems, and the possession of defence mechanisms capable of dealing with soluble foreign materials.

4.1.4 ZINC IN CADMIUM METABOLISM

Zinc aids in protection against cadmium challenge in some vertebrates (Friberg, et al., 1974) probably through the induction of thionein synthesis. No studies have examined the role of zinc in protection of marine invertebrates against cadmium challenge. However, Fowler and Benayoun (1974) have examined the effect of zinc on the accumulation of cadmium. In *Mytilus galloprovincialis* zinc (30 and 100 µg/l) did not affect the rate of accumulation of ^{109}cadmium (approximately 0.15 µg/l) by the whole animal over 13 days, and did not alter the organ distribution of ^{109}cadmium.

Zinc, whilst chemically similar to cadmium, need not necessarily be accumulated by either the same molecular mechanism or the same route as cadmium. The National Health and Medical Research Council noted an "apparent positive correlation between zinc and cadmium present in oysters" (Australian Department of Health, 1975), although it is not clear whether such an "apparent correlation" is a result of, or reflects, environmental levels, or whether there is some further biological factor involved.

Ratkowsky, et al. (1974) examined zinc and cadmium in oysters from both polluted and unpolluted Tasmanian waters, and the zinc/cadmium ratio (µg/g wet weight, mean of ten animals/location) ranged from 110 to 2292 in samples from 48 locations. Further, Ratkowsky, et al. (1974) identified three major groups of locations by

numerical techniques, those "near" (C), "in between" (B) and "far" (A) from urban/industrial areas. The zinc/cadmium ratios for these groups are shown in Table 4.1.

TABLE 4.1

Zinc/cadmium ratios in Oysters*.

	A (far)	B (in between)	C (near)
Range	114-2292	161-925	110-625

* data recalculated from Ratkowsky, et al., 1974.

Mackay, et al. (1975) and Hugget, Bender and Slone (1973) have reported that oysters from non-polluted areas display a linear relationship between the levels of zinc and cadmium. However, Hugget, Bender and Slone (1973) also show that the cadmium level in oysters from polluted areas is not related to their zinc level, and it seems likely that the linear relationship in unpolluted oysters reflects environmental levels, rather than other factors (such as stoichiometric linear relation in uptake).

In addition, Romeril (1971) discusses some evidence in favour of different binding sites for cadmium and zinc. In view of the differences in stability constants of some common biological ligands for cadmium and zinc, and their response to changes in ionic environment (Vallee and Ulmer, 1972), and the above discussion, it seems unlikely that zinc and cadmium have identical mechanisms of accumulation.

In summary, there appears to be no evidence to relate the mechanism, the route, or the level of cadmium accumulation to that of

zinc in oysters. This conclusion is in contrast to the commonly held view in the literature that cadmium may be absorbed by organisms because of its chemical similarity to zinc (e.g. Friberg, et al., 1974, p. 51).

4.1.5 BIOLOGICAL HALF TIME

A parameter often employed to assess the stability of an accumulated body burden of metal is the biological half time, which can also be conceptually related to the turnover of tissue bound metal. In the human kidney the biological half time ($t_{\frac{1}{2}}$) for cadmium has been estimated to be between 17.6 and 33 years (Task Group on Metal Accumulation, 1973). However, the $t_{\frac{1}{2}}$ for cadmium in other terrestrial animals may range from seven days (an insect) to 99 days (a bird, a spider) (Van Hook, et al., 1974).

In aquatic animals, Fowler and Benayoun (1974) have estimated a $t_{\frac{1}{2}}$ for cadmium in *Mytilus galloprovincialis* of 307 to 1254 days, (depending probably on food supply) and in a benthic shrimp, (*Lysmata seticaudata*) one of 378 days.

The probably long $t_{\frac{1}{2}}$ for cadmium in oysters means that, for an accurate assessment, a long loss experiment needs to be conducted. Such an experiment to determine biological half time was not conducted. Brief experiments can give erroneous results for $t_{\frac{1}{2}}$ because of possible multi-compartmental losses of metal.

The assessment of organ loads by atomic absorption spectroscopy is not capable of displaying any redistribution of cadmium between the organs which may occur as cadmium levels increase. To assist in the qualitative assessment of the stability of cadmium loads

within the organs (in the absence of $t_{\frac{1}{2}}$ data), a pulse labelling technique was employed. The technique substitutes a pulse of radioactive cadmium for the "cold" cadmium, and following a period of time organs are examined for distribution of radioactive cadmium. Essentially, such a technique follows the fate of cadmium accumulated over a particular brief interval of time.

This chapter describes an experimental approach to the quantitative assessment of organ loads of cadmium at several ambient levels of cadmium. The accumulation is followed over the 16 week experimental period, and the acute toxicity of cadmium at the higher concentrations is noted. A peripheral study relating to the stability of the organ loads of cadmium using a whole body autoradiographic technique is also described.

4.2 METHODS

Oysters were purchased locally. The source (verified at each purchase by inspection of the oyster sack) was the Clyde River, Batemans Bay, N.S.W. The Clyde River drains a large area of forest, with urban development within the catchment being restricted to the town of Batemans Bay at the mouth and the village of Nelligen several kilometres upstream. Apart from these urban areas, a sawmill and some recreational and pleasure boating, there are no other land uses in the catchment likely to contribute significant amounts of cadmium to the river. Accordingly, the cadmium levels in oysters from the Clyde River are relatively low (0.2µg/g by wet weight, Mackay, et al., 1975).

Oyster farming in the Clyde is well established along traditional N.S.W. lines (the stick and tray technique), and both the

proximity of the river to Canberra and the relatively unpolluted nature of the water make the oysters from the area well suited to this study.

The flowing system (Chapter 3) was used to expose the oysters to cadmium. Sea water was transported to Canberra at weekly intervals in polyethylene tanks and stored outside at ambient temperature, with aeration. The pumping system and pipes, although all plastic, were soaked for long periods in sea water prior to use. Sea water was collected at the N.S.W. State Fisheries boatshed near the mouth of the Clyde River, by courtesy of the Director of Fisheries, and with some assistance from the local officer, Mr K. Cree.

4.2.1 ORGAN ACCUMULATION OF CADMIUM

Three hundred oysters were scrubbed with a nylon bristled brush to remove epiphytes, and acclimated for four weeks to laboratory conditions in five experimental tanks (60/tank, allotted at random). At the commencement of the experiment each tank received supplies of cadmium chloride/sea water solution and sea water sufficient to maintain a final concentration of approximately 10, 25, 50 and 150 µg/l, while the control tank received sea water only. A constant temperature ($20\pm2^{o}C$) and a 12/12 light dark regime was maintained. At the sampling times of 0, 1, 4, 8 and 16 weeks 12 oysters were removed from each tank. Two oysters were used for autoradiography (to be described later) and the remaining ten oysters were shucked and their soft parts divided into mantle, gill, muscle, visceral mass and heart/kidney. Samples of haemolymph were extracted, although there may have been some contamination with pericardial fluid and sea water. Clean stainless steel instruments were used throughout. All organs were stored at $-17^{o}C$ in polypropylene tubes which had been soaked in 5%

nitric acid.

The organ designated "heart/kidney" is composed mostly of heart tissue with a small component of excretory organ closely associated. The major component of the excretory organ could not be extracted without undue difficulty, since it is a thin membranous organ lying partially within the visceral mass at the pyloric process, close to the adductor muscle. Biopsy was considered for sampling the excretory organ, but discarded because of problems of repeatability, and because of the time and contamination factors involved.

After storage each organ was assayed individually for cadmium by atomic absorption spectroscopy (AAS). All background tissues were analysed using a graphite rod atomizer (see Appendix 1 for further details), while other levels were high enough to allow the use of flame AAS.

Tissue was prepared for analysis by rinsing (with distilled deionized water) the stored organ into a lidded nickel crucible. After drying to constant weight (24 hours at 80^{o}C) the organs were ashed at 450^{o} for 24 hours. Re-ashing was not necessary since in almost all cases a clean white ash was obtained. After cooling, 1 ml of distilled deionized water was added to the crucible, followed by 1 ml ARISTAR (BDH analytical grade) nitric acid. The solution was mixed and returned to the storage tube. Eight ml of distilled deionized water was pipetted into the crucible and allowed to stand for one min, and then finally poured into the storage tube to achieve a final volume of 10 ml. In two trials recovery of added cadmium from a spiked sample averaged 93%. Assays were not corrected for percentage recovery. The sample preparation techniques were similar to those of Nielsen (1974),

Nielsen and Nathan (1975) and Zaroogian and Cheer (1976). Nielsen and Nathan (1975) report 100% recovery of cadmium from their technique.

Standard curves prepared on the carbon rod atomizer were not linear and did not follow any simple expression over the range 1 µg/l-250 µg/l. A computer program was written to allow accurate interpolation of the unknowns into the standard curve from the carbon rod. Standard curves prepared by flame AAS were linear over the range 0.1 mg/l-2.5 mg/l, but the computer program was also used for these values. Cadmium standard solutions were stored in polyethylene bottles at $4^{o}C$. Soaking of the bottles in 10% nitric acid for at least one week was found to be critical for the compilation of accurate standard curves, especially at the lower levels.

Test solutions were, in some cases, diluted 1/5 for analysis by flame AAS, using, in all cases, distilled deionized water and acid soaked tubes. Further details of the AAS procedures are included in Appendix 1.

The results of the analyses are expressed at µg Cd/g dry weight oyster tissue. The conversion factor for dry weight to wet weight is approximately 5 (see Appendix 2).

4.2.2 AUTORADIOGRAPHY

At each sampling time two oysters were exposed for four hours to ^{109}Cd in sea water at the respective cadmium concentration. All oysters were observed to be feeding during this period. 0.5 µCi $^{109}CdCl_2$ (Amersham, essentially carrier free) in 500 ml unfiltered sea water was employed as a "pulse" of cadmium, followed by a "cold chase" of unlabelled cadmium chloride (at the respective

cadmium level) for 72 hours. Sea water was changed each 24 hours up to 72 hours when both oysters were shucked and frozen immediately in a carbon dioxide freezing chamber, then stored at -20°C. The duplicate oysters were sectioned in a freezing microtome unit at -18°C with no further fixation or preservation, and 100 µm sections were taken up on glass slides and dried in the hot air blast from a hair drier.

Sections were apposed to X-ray film ("Kodirex" 5x7) for eight weeks at -17°C, and developed to maximum fog level (five min at 20°C) in Kodak X-ray Developer type 2. Impressions on the X-ray film were photographed and the positive is reproduced here. Blanks of oyster tissue (free from radioactivity) leave no impressions on the X-ray film. After exposure to the film the slides were stained, mounted and dried. The staining schedule is as follows:

10% formalin, 20 min minimum

1% Azure Blue II, four min

Rinse in water

70% ethanol, two min

0.5% Eosin in 70% ethanol, two min

70% ethanol, one min

Saturated Orange G in 90% ethanol, three min

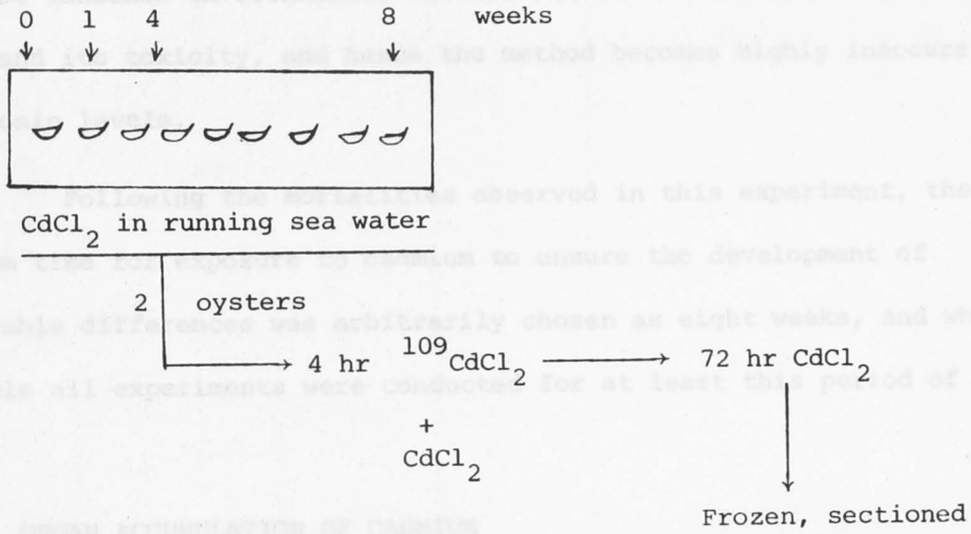
90% ethanol, six min

100% ethanol, ten min

Xylene, 20 min, mount and dry.

The protocol for the autoradiography experiments is presented diagramatically below.

Protocol for autoradiography:



4.3 RESULTS

4.3.1 MORTALITIES

Significant mortality is caused by 150,50 and 25 µg/l cadmium over 60 days (Figure 4.1). Control and 10 µg/l groups show some mortality, and, in the control group, this may be attributable to the low level of food particles present in the flowing sea water system. The toxicity of cadmium may be influenced by the lack of food. However, over the 16 week experimental period, 10 µg/l appears to be a sub-lethal level of cadmium, since mortality is not different from that of the controls.

It is not possible to directly predict the LC50, or predict an incipient lethal level, because extrapolations from the observed rates of die off are not valid. The sampling procedure was continually biased by the removal of living animals for the accumulation analyses, ensuring that only longer-lived animals remained at the later times.

Further, although it is possible to plot LT50s against concentration to predict incipient lethal levels, such an approach suffers in that it does not consider environmental factors and their interaction with the metal and its toxicity, and hence the method becomes highly inaccurate at chronic levels.

Following the mortalities observed in this experiment, the minimum time for exposure to cadmium to ensure the development of measurable differences was arbitrarily chosen as eight weeks, and where possible all experiments were conducted for at least this period of time.

4.3.2 ORGAN ACCUMULATION OF CADMIUM

4.3.2.1 WHOLE BODY ACCUMULATION - RESULTS AND DISCUSSION

The accumulation of cadmium by oysters at each concentration is shown in Figure 4.2. The results of the organ assays are shown in Table 4.3, and the whole body values of Table 4.2 have been computed by adding the amount of cadmium in the organs of each oyster, and dividing by the dry weight of the organs. The cadmium concentrations of the heart/kidney have been omitted from the results in Table 4.2 since the probable large errors involved with the determination make the values unreliable. The pooled heart/kidneys usually weighed less than 15 mg dry weight, and there is no estimate of inter-animal variation.

The concentration of cadmium in oysters in the 25 and 50 µg/l groups appeared to plateau (Figure 4.2 and Table 4.2), while the concentrations in the 150 µg/l group did not display a plateau before all the oysters died. The concentration of cadmium in the oysters from the 10 µg/l and control groups did not approach a plateau over the 16 week period. The significance of the plateau is unknown, but it is

FIGURE 4.1

The cumulative mortality of oysters exposed to 0 (control), 10, 25, 50 and 150 µg/l cadmium in the flowing sea water system. The results were collected by observing the mortality (on a daily basis) of the oysters used for the 16 week accumulation experiment.

FIGURE 4.2

The accumulation of cadmium by whole oysters exposed to 0 (control), 10, 25, 50 and 150 µg/l cadmium in the flowing system. The sum of the amounts of cadmium in each organ (except the heart/kidney) was divided by the sum of the dry weight of the organs of each oyster, to give the concentration of cadmium in each oyster (calculated from the results in Table 4.3). The points are means $\pm$ s.e.m. of five to ten observations. The r values for the regressions for the 10 µg/l and the control group indicate a good fit to a straight line. The 25, 50 and 150 µg/l groups cannot be accurately described by a linear regression.

controls $y = 2.4\ x + 5.7$ ($r = 0.766$)

10 µg/l $y = 7.4\ x + 6.9$ ($r = 0.906$, $P < 0.05$)

FIGURE 4.1

MORTALITIES OF OYSTERS EXPOSED TO CADMIUM

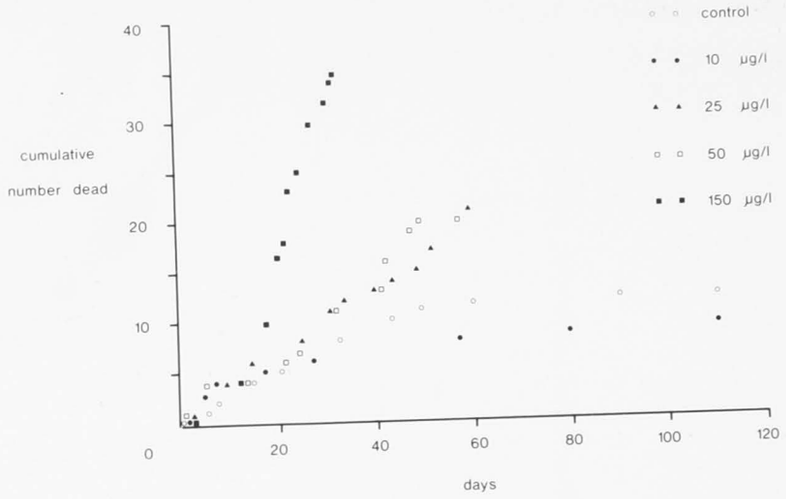


FIGURE 4.2

ACCUMULATION OF CADMIUM BY WHOLE OYSTERS

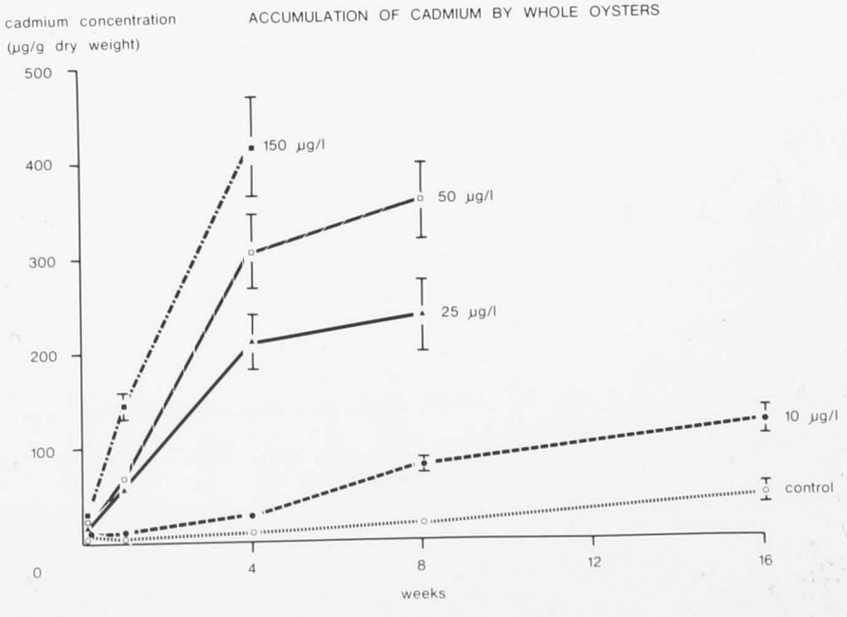


TABLE 4.2

Concentration (µg/g) and amount (µg) of cadmium per oyster, in oysters exposed to five levels of cadmium in sea water. Mean ± s.e.m.

	Control		10 µg/l		25 µg/l		50 µg/l		150 µg/l	
Week	µg/g	µg	µg/g	µg	µg/g	µg	µg/g	µg	µg/g	µg
0	10.9± 1.7	7.6			(composite sample from all groups)					
1	5.6± 0.5	3.7	12.0± 1.6	8.0	55.9± 4.0	48.5	67.0±13.3	52.6	147.1±14.2	120.9
4	12.5± 1.3	7.7	29.2± 5.6	20.7	212.4±29.5[b]	139.1	307.8±39.6[c]	265.2	421.6±53.3	290.2
8	21.6± 1.9	11.3	81.7± 7.6[a]	40.8	237.8±37.5[b]	130.5	359.0±40.5[c]	203.5	-	
16	46.7±11.2	24.2	123.1±15.0[a]	54.7	-		-		-	

a, b, c Pairs of concentrations marked with the same letter are not significantly different (t test)

(-, not assayed)

unlikely to represent an equilibrium or steady state condition associated with tolerance to the metal, since both groups reach 100% mortality soon after. Indeed, the plateau may indicate saturation of available binding sites, and imminent death. All oysters sectioned for autoradiography (4.2.2) showed abundant gonad tissue, so spawning cannot be used to explain the plateau in oyster concentrations.

The regression of cadmium concentration on time is linear for the results from the 10 µg/l group of oysters ($y = 7.4 x + 6.9$, $r = 0.906$, $p < .05$). The results from the 25 and 50 µg/l groups do not adequately fit a linear expression, the exponential curves of the form proposed by Shuster and Pringle (1969) and Pentreath (1973), or any other simple curvilinear expression. In some circumstances the $t_{\frac{1}{2}}$ can be estimated from accumulation data. The conditions and method are described in Pentreath (1973), but in essence depend upon the accumulation curve approaching an asymptote, and upon a suitable mathematical model being available to describe the loss of metal.

Pentreath (1973) studied the elimination of isotopes of Zn, Mn, Fe and Co from the organs of *Mytilus edulis*, and found that a simple exponential model adequately described all the losses. Hess, Smith and Price (1975) employed a similar model in an examination of the accumulation and loss of a number of metals in oysters. However, the literature does not provide any satisfactory models for the loss of cadmium from oysters. Since the accumulation curves for cadmium in *C. commercialis* do not adequately fit the accumulation model proposed by Pentreath (1973) it is not possible to estimate $t_{\frac{1}{2}}$ using that method.

The concentration of cadmium in control oysters and those

exposed to 10 µg/l both increase slowly, reaching 44 and 124 µg/g respectively after 16 weeks. The fourfold increase in concentration in the controls indicates trace contamination of sea water used in the experimental system. Both groups of oysters reach concentrations of cadmium substantially higher than the maximum recommended levels for cadmium in oysters (2 µg/g wet weight, approximately 10 µg/g dry weight), (Australian Department of Health, N.H and M.R.C., 1975). The increase in the cadmium concentration in the control group is associated with an increase in metal levels and not simply a drop in dry weight (Table 4.2).

Zaroogian and Cheer (1976) found that *C. virginica* was able to accumulate approximately 22 µg/g of cadmium over four weeks (at near 20°C) from 5 µg/l cadmium in a flowing sea water system. That value compares favourably with the approximately 30 µg/g per four weeks from 10 µg/l at a similar temperature, reported here.

Mackay, et al. (1975) have reported a level of cadmium of 0.2 µg/g (wet weight) (approximately 1 µg/g dry weight) from oysters from the Clyde River. One oyster from the Clyde River prepared by the methods described in section 4.2.1 has been independently analysed by Dr J.B. Willis (Division of Chemical Physics, C.S.I.R.O., Clayton, Victoria) and found to have a level of 2.5 µg/g dry weight cadmium. Experimental oysters sampled at week 0 had a mean level of near 11 µg/g cadmium, and the level of cadmium in the control oysters continued to increase over the experimental period. The discrepancy between the figures above is probably caused by accumulation of cadmium by the oysters during the four week acclimation period, since the water used during acclimation was collected and handled in the same manner as the

water for the period of exposure to cadmium.

Oysters (*C. virginica*) exposed to 100 and 200 µg/l cadmium in a flowing system do not accumulate more than approximately 500 µg/g (dry weight) (Shuster and Pringle, 1969). Heavy mortalities occur at that level and terminate experiments. *C. commercialis* exposed to 150 µg/l cadmium accumulates cadmium up to a level which is similar (422 ± 53 µg/g, Table 4.2) before mortalities terminate the experiment. The maximum level was attained by *C. commercialis* between four and eight weeks, compared with approximately 14 weeks for *C. virginica* (Shuster and Pringle, 1969). The different experimental conditions, and possibly different size and age oysters, may explain the apparent difference in the rate of accumulation by the two species of oyster.

4.3.2.2 DISTRIBUTION OF CADMIUM BETWEEN THE ORGANS - RESULTS AND DISCUSSION

The complete results of organ assays are shown in Table 4.3.

The accumulation of cadmium by the organs, at each concentration, is plotted in Figures 4.3 to 4.12.

At all elevated concentrations of water cadmium (that is, not the control group) the gill contains the highest concentration of cadmium.

The regression of cadmium concentration (y) on time (x) for each organ in the 10 µg/l group gives a good fit to a linear expression:

visceral mass $y = 8.78x + 8.61$ ($r = 0.88$, $p < 0.05$)

mantle $y = 5.45x + 7.83$ ($r = 0.83$)

gill $y = 9.63x + 6.36$ ($r = 0.84$)

muscle $y = 6.41x + 0.07$ ($r = 0.85$).

The fit to a straight line is less reliable for the organs of the control group of oysters:

visceral mass $y = 3.62x + 6.56$ ($r = 0.83$)

mantle $y = 3.13x + 3.56$ ($r = 0.57$)

gill $y = 1.12x + 8.52$ ($r = 0.69$)

Regressions of y on x for organs from oysters in the 25 and 30 µg/l groups cannot be reliably described by a linear expression.

The accumulation of lead by organs of *C. virginica* (Pringle, et. al., 1968) is shown in Figure 4.13, together with the data for cadmium plotted in a similar manner in Figure 4.14. The accumulation curve for lead in *C. virginica* is strikingly different from that for cadmium in *C. commercialis*.

The non-linear accumulation (Figure 4.14) and the failure of the organs (and the whole oyster) in the 10 and 150 µg/l groups to reach a plateau of cadmium concentration mean that it is not possible to correlate body loads at any time with previous exposure to a particular level of cadmium, in other than a qualitative manner. Given a longer exposure than 16 weeks the oysters of the 10 µg/l group may have reached a plateau. The results in Table 4.2 indicate that this may have been reached at 16 weeks, but more information is needed before this trend can be confirmed.

If oysters can achieve a steady state between cadmium in their tissues and cadmium in the water, body loads of cadmium in

FIGURE 4.3

The accumulation of cadmium by the visceral mass from oysters exposed to 0 (control), 10, 25, 50 and 150 µg/l cadmium over 16 weeks in the flowing sea water system. The points are the means ± s.e.m., using the results from Table 4.3.

FIGURE 4.4

The accumulation of cadmium by the mantle from oysters exposed to 0 (control), 10, 25, 50, and 150 µg/l cadmium over 16 weeks in the flowing sea water system. The points are means ± s.e.m., using the results from Table 4.3.

FIGURE 4.5

The accumulation of cadmium by the gill of oysters exposed to 0 (control), 10, 25, 50 and 150 µg/l cadmium over 16 weeks in the flowing sea water system. The points are means ± s.e.m., using the results from Table 4.3.

FIGURE 4.3

ACCUMULATION OF CADMIUM BY THE VISCERAL MASS

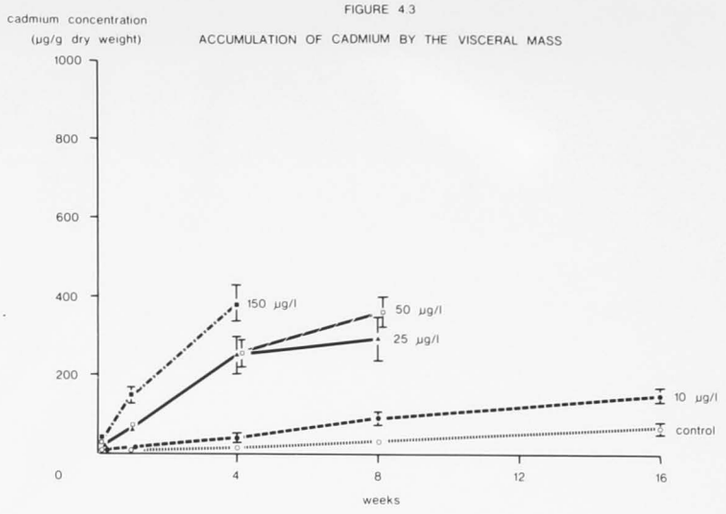


FIGURE 4.4

ACCUMULATION OF CADMIUM BY THE MANTLE

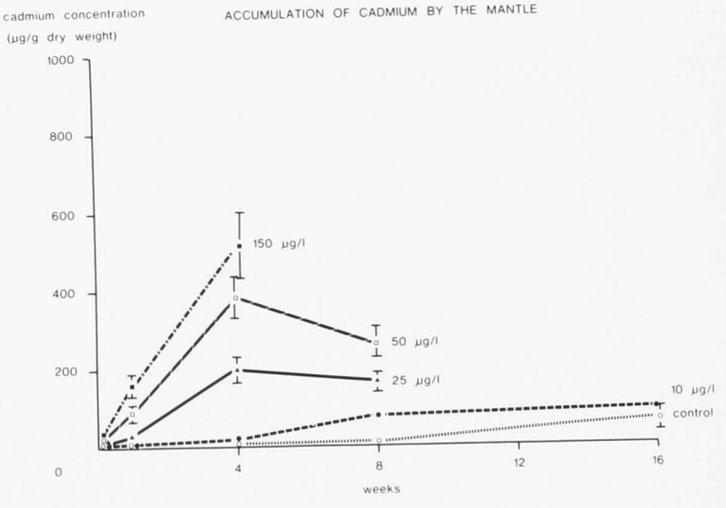


FIGURE 4.5

ACCUMULATION OF CADMIUM BY THE GILL

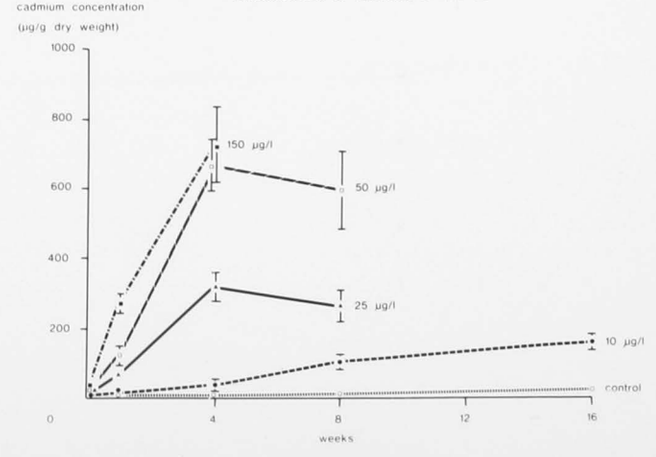


FIGURE 4.6

The accumulation of cadmium by the muscle of oysters exposed to 0 (control), 10, 25, 50, and 150 µg/l cadmium over 16 weeks in the flowing sea water system. The points are means ± s.e.m., using the results from Table 4.3.

FIGURE 4.7

The accumulation of cadmium by heart/kidney tissue pooled from oysters exposed to 0 (control), 10, 25, 50 and 150 µg/l cadmium over 16 weeks in the flowing sea water system. The points are single assays of the cadmium concentration in the pooled tissue. The results are from Table 4.3.

FIGURE 4.6

ACCUMULATION OF CADMIUM BY THE MUSCLE

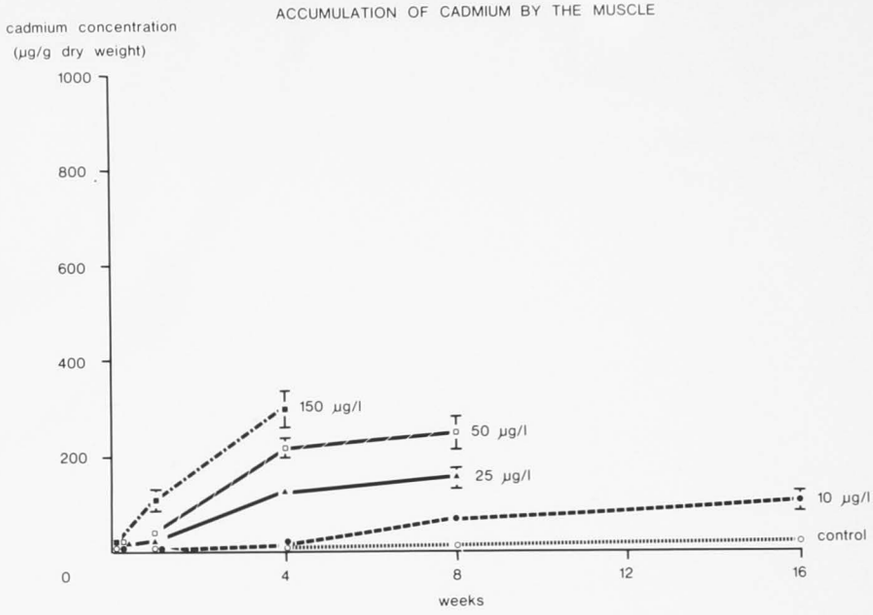


FIGURE 4.7

ACCUMULATION OF CADMIUM BY THE HEART/KIDNEY

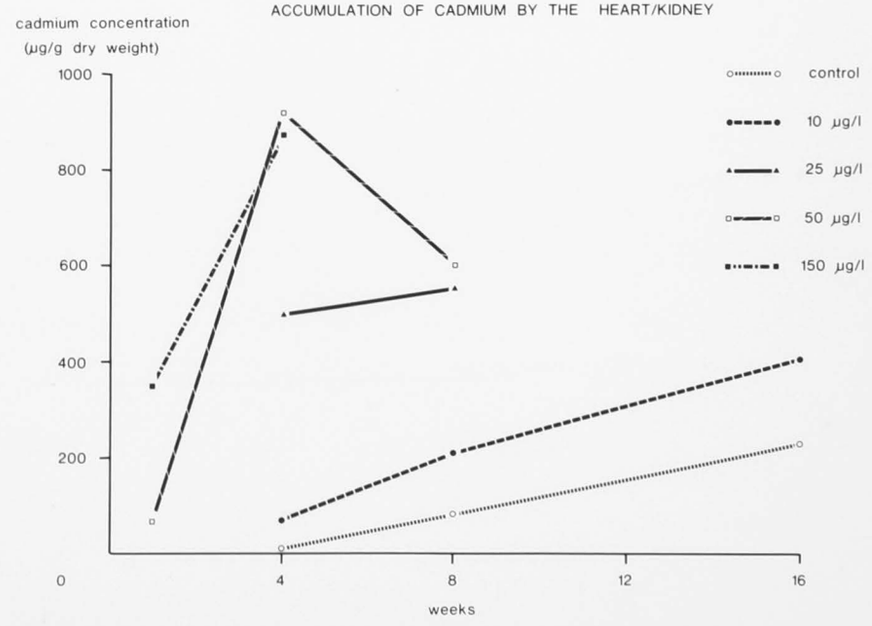


FIGURE 4.8

The concentration of cadmium in organs from oysters not treated with cadmium (control). The points are means ± s.e.m., using the results from Table 4.3.

FIGURE 4.9

The accumulation of cadmium by organs of oysters exposed to 10 µg/l cadmium. The concentration factor (level of cadmium in tissue [µg/g]/level of cadmium in water [µg/l]) is plotted against time of exposure. The points are means ± s.e.m., using the results from Table 4.3.

FIGURE 4.10

The accumulation of cadmium by organs of oysters exposed to 25 µg/l cadmium. The concentration factor (see Figure 4.9) is plotted against time of exposure. The points are means ± s.e.m., using the results from Table 4.3.

FIGURE 4.8

THE CONCENTRATION OF CADMIUM IN OYSTER ORGANS (control)

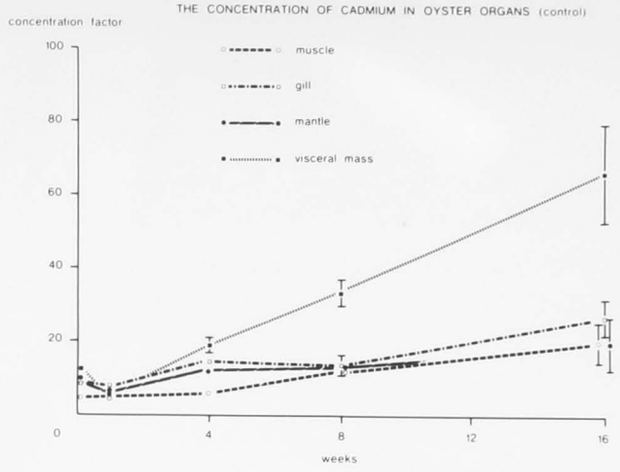


FIGURE 4.9

ACCUMULATION OF CADMIUM BY OYSTER ORGANS (10 µg/l)

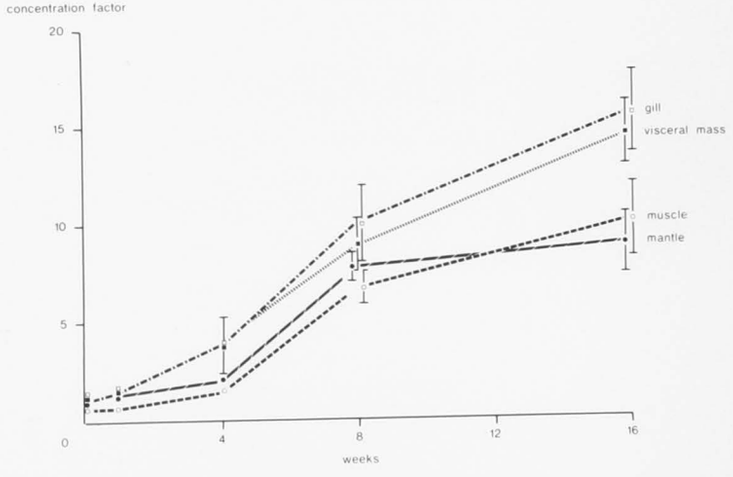


FIGURE 4.10

ACCUMULATION OF CADMIUM BY OYSTER ORGANS (25 µg/l)

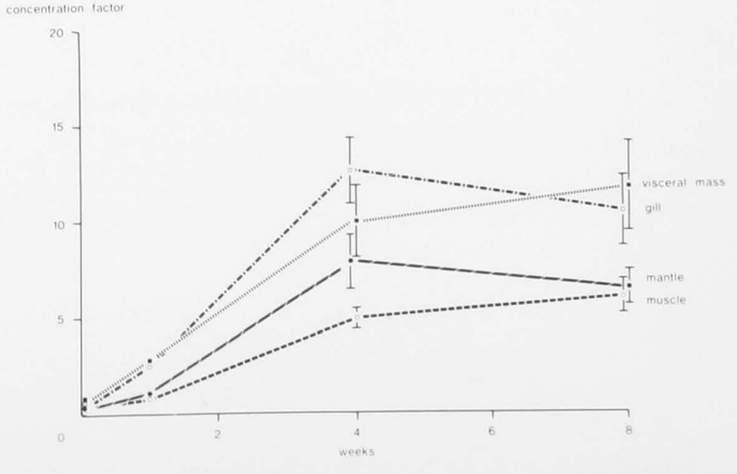


FIGURE 4.11

The accumulation of cadmium by organs of oysters exposed to 50 µg/l cadmium. The concentration factor (see Figure 4.9) is plotted against time of exposure. The points are means ± s.e.m., using the results from Table 4.3.

FIGURE 4.12

The accumulation of cadmium by organs of oysters exposed to 150 µg/l cadmium. The concentration factor (see Figure 4.9) is plotted against time of exposure. The points are means ± s.e.m., using the results from Table 4.3.

FIGURE 4.11

ACCUMULATION OF CADMIUM BY OYSTER ORGANS (50 µg/l)

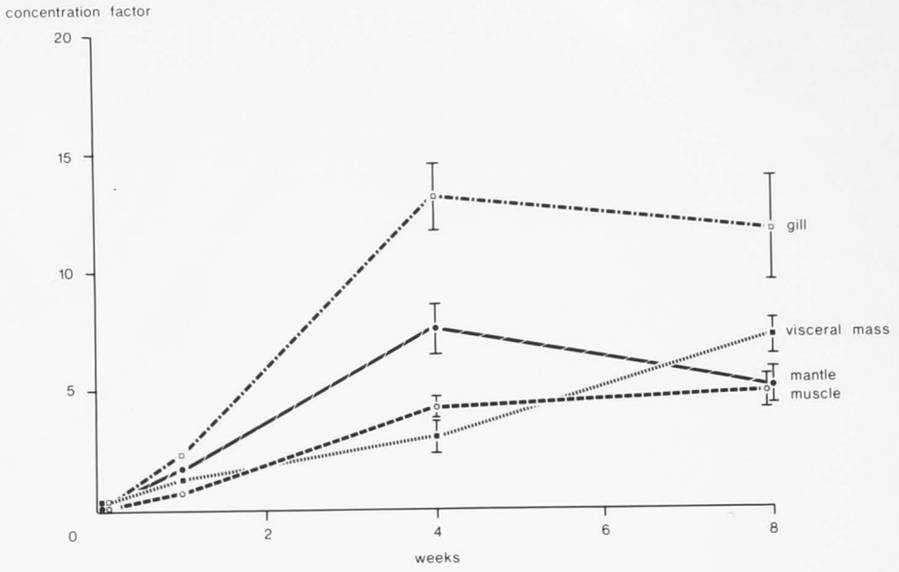


FIGURE 4.12

ACCUMULATION OF CADMIUM BY OYSTER ORGANS (150 µg/l)

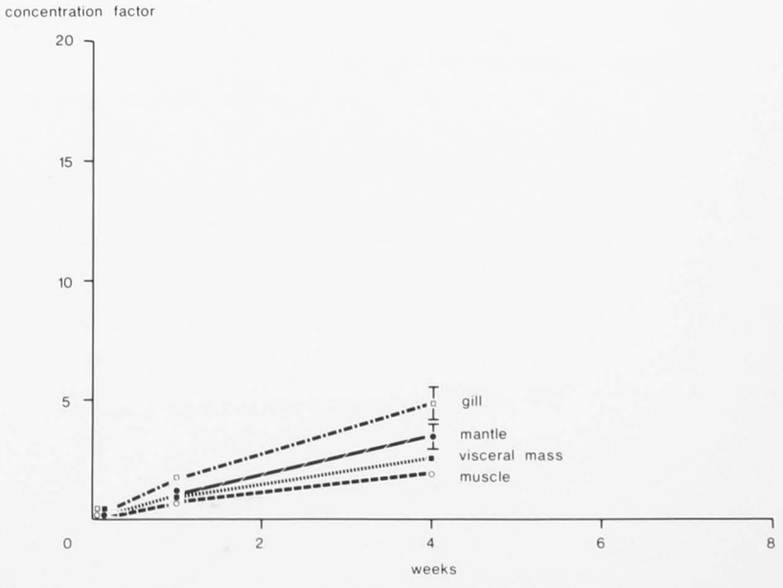


FIGURE 4.13

The accumulation of lead by organs of *C. virginica* after seven weeks exposure to four concentrations of lead in sea water. The data is redrawn from Pringle, et al. (1968).

FIGURE 4.14

The accumulation of cadmium by the organs of *C. commercialis* after four weeks exposure to four concentrations of cadmium. The points are means $\pm$ s.e.m.

FIGURE 4.13

ACCUMULATION OF LEAD BY C. virginica

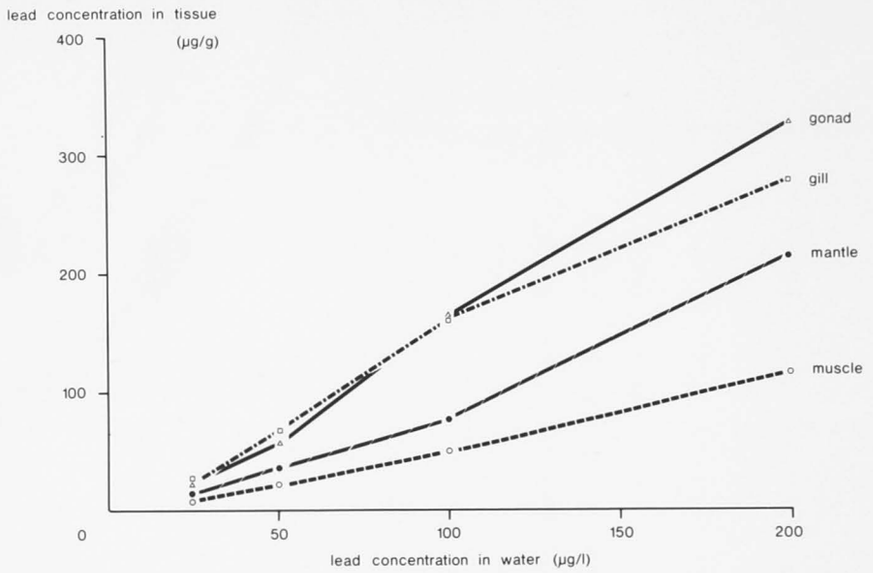


FIGURE 4.14

ACCUMULATION OF CADMIUM BY C. commercialis

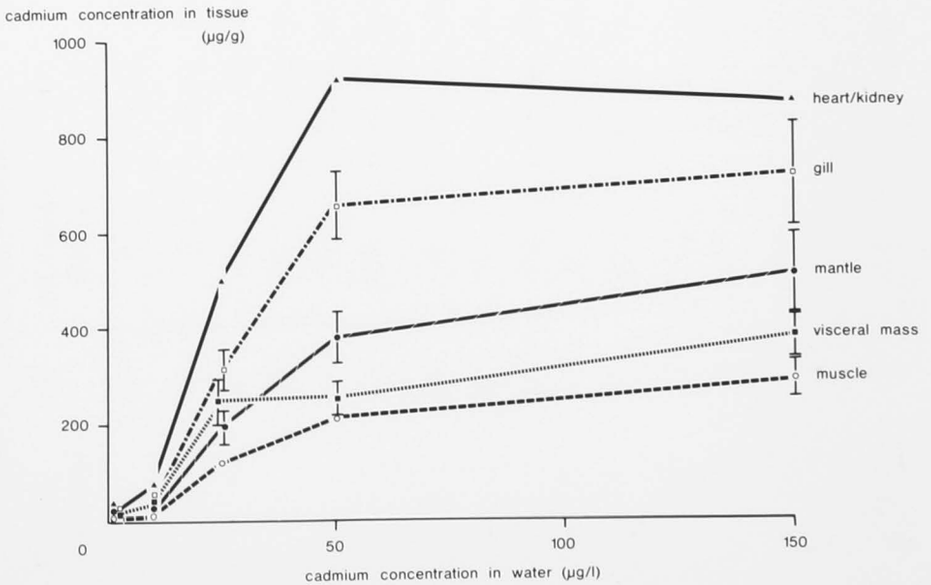


TABLE 4.3

Cadmium concentrations in oyster organs after a 16 week exposure to five levels of cadmium (µg/g dry wt). Mean ± s.e.m. N = no. of organs per mean; V = visceral mass; Ma = mantle; G = gills; Ms = muscle; H = heart/kidney; Bl = blood; - = not assayed; 0 µg/l = control.

Time (Weeks)	N	Cd Level (µg/l)	Organs V	Ma	G	Ms	H	Bl
0	10	0	13.5± 2.4	9.4± 1.3	9.6± 2.0	5.6± 1.1		
1	10	0	5.4± 0.6	6.4± 1.2	7.9± 0.9	4.7± 1.3	-	-
	10	10	13.1± 2.0	12.7± 2.3	13.8± 2.8	6.0± 1.0	-	-
	10	25	70.2± 6.3	29.0± 2.6	63.1± 7.2	22.0± 3.8	-	-
	10	50	63.1± 14.2	89.9± 22.3	119.1± 27.7	40.0± 6.8	69.6	-
	10	150	150.3± 19.6	157.0± 26.2	266.7± 26.5	106.5± 20.9	350.4	-
4	10	0	19.2± 2.1	12.75± 2.2	14.8± 1.6	6.5± 0.6	15.6	9.3
	10	10	39.5± 10.6	19.5± 2.9	37.5± 15.1	14.0± 1.7	72.9	8.7
	10	25	248.3± 46.7	197.5± 34.1	316.6± 41.4	122.7± 12.6	500.0	12.4
	10	50	253.3± 34.8	383.7± 52.9	659.6± 71.6	215.6± 19.1	917.2	-
	10	150	381.6± 45.5	512.1± 84.0	719.9±107.2	292.5± 38.4	869.4	169.6
8	5	0	33.5± 3.7	13.3± 2.2	14.1± 2.8	12.3± 2.1	-	-
	5	10	89.9± 15.0	77.7± 7.5	100.5± 19.4	67.1± 8.2	213.8	-
	6	25	291.2± 57.4	162.3± 23.3	260.5± 44.3	150.3± 21.5	553.0	-
	6	50	361.4± 38.4	259.7± 37.7	589.5±109.4	246.2± 34.8	601.0	-
16	6	0	66.4± 13.5	61.0± 26.4	27.3± 5.0	20.6± 5.4	224.6	-
	7	10	147.0± 16.4	89.3± 15.6	157.0± 21.2	101.4± 19.2	404.6	-

TABLE 4.4

Dry weight of organs (mg), mean ± s.e.m. Other information as in Table 4.3.

Time (Weeks)	Cd Level (µg/l)	V	Ma	G	Ms	H
0	0	425.7±68.9	88.8±11.2	81.9± 9.3	102.7±15.0	-
1	0	415.1±45.2	101.3±15.1	69.5± 7.5	72.8±12.4	-
	10	396.3±50.1	91.8±11.6	86.7±10.0	90.9± 9.8	-
	25	506.6±53.8	134.2±13.2	122.6± 7.0	104.3± 7.8	14.7
	50	514.4±37.1	93.5±14.0	92.2± 9.9	85.1± 6.0	11.5
	150	473.2±42.0	120.7±20.3	94.2± 8.8	134.1±27.3	13.7
4	0	264.6±39.4	117.6± 7.6	115.3± 4.0	120.9± 9.1	16.7
	10	344.9±41.8	126.8±14.8	111.2± 9.8	123.4±13.1	16.6
	25	313.2±39.1	137.2±16.7	98.9± 8.0	105.5± 6.4	14.5
	50	541.8±21.8	111.3± 9.3	93.2± 6.6	115.2± 9.2	16.9
	150	411.5±25.9	93.7± 8.5	78.5± 5.3	104.5± 9.0	19.9
8	0	211.2±48.2	115.5±14.7	93.8± 8.2	100.7±13.9	
	10	206.0±56.1	105.5± 7.6	90.8± 9.3	99.6± 8.2	15.9
	25	272.8±28.3	85.1± 8.0	84.5± 7.2	106.4±12.1	16.0
	50	272.8±47.8	104.2±13.9	87.5± 9.9	102.5±14.7	13.8
16	0	206.7±47.8	108.9±13.5	88.8± 5.5	113.3± 8.1	11.4
	10	147.9±23.2	110.9±14.7	93.2±13.5	92.2±11.0	15.2

oysters from natural situations could be used to predict water levels. Since, at 10 µg/l, a plateau is not reached, the body load of cadmium in oysters from field situations is of little use in predicting past exposure to cadmium. A more appropriate parameter may be the instantaneous accumulation rate, although there appears to have been little discussion of this in the literature, and no studies have been attempted on natural populations.

Table 4.5 describes the absolute amount of metal in each organ as a percentage of the total for all organs (equivalent to whole oyster) at weeks 0, 1, 4, 8.

Despite the high concentrations observed in the heart/kidney (Table 4.2) only a small percentage of the total metal in the oyster is present in the heart/kidney (Table 4.5).

Other than the controls and the 10 µg/l group, the organ concentrations are generally in the order gill > viscera $\cong$ mantle > muscle (Table 4.3), and in the 50 and 150 µg/l groups the relative position of the mantle and visceral mass appear to change with time. In the control group the concentration in the visceral mass is consistently higher than the other organs and the pattern is generally viscera > gill $\cong$ mantle > muscle. In the 10 µg/l group the pattern is viscera $\cong$ gill > mantle > muscle. The gill changes its position in the ranking of the organs, apparently in response to increasing water levels of cadmium, suggesting a role for the gill in direct accumulation of cadmium from the water.

The plateau observed for the accumulation of cadmium by whole oysters exposed to 25 and 50 µg/l cadmium (Table 4.2) is reflected by

FIGURE 4.15

The dry weight of the organs of oysters from the flowing sea water system. The oysters were not exposed to cadmium. The points are means ± s.e.m., using the results from Table 4.4.

FIGURE 4.16

The dry weight of the organs of oysters exposed to 10 µg/l cadmium for 16 weeks. The points are means s.e.m., using the results from Table 4.4.

FIGURE 4.17

The dry weight of the organs of oysters exposed to 25 µg/l cadmium for eight weeks. The points are means ± s.e.m., using the results from Table 4.4.

FIGURE 4.15

DRY WEIGHT OF ORGANS (control)

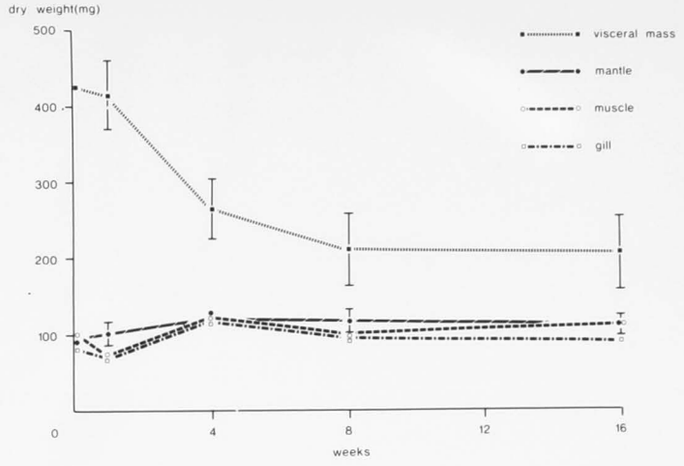


FIGURE 4.16

DRY WEIGHT OF ORGANS (10 µg/l)

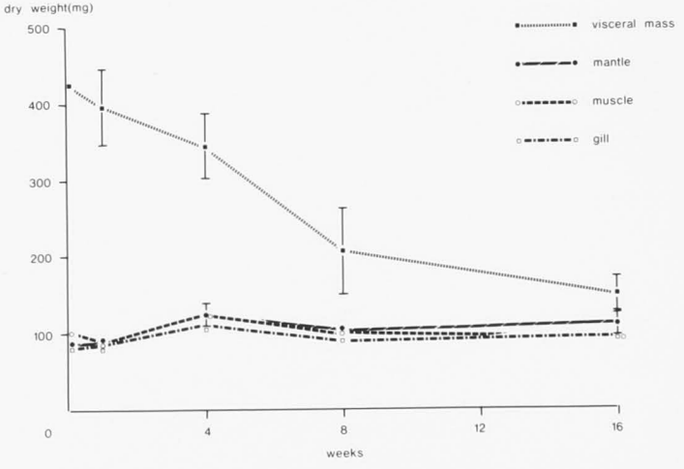


FIGURE 4.17

DRY WEIGHT OF ORGANS (25 µg/l)

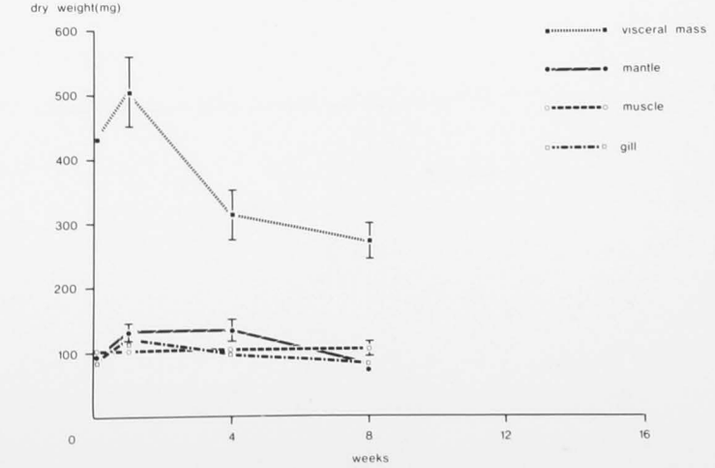


FIGURE 4.18

The dry weight of the organs of oysters exposed to 50 µg/l cadmium for eight weeks. The points are means ± s.e.m., using the results from Table 4.4.

FIGURE 4.19

The dry weight of the organs of oysters exposed to 150 µg/l cadmium for four weeks. The points are means ± s.e.m., using the results from Table 4.4.

FIGURE 4.20

The accumulation of cadmium by whole oysters. The concentration factor (see Figure 4.9) for accumulation of cadmium from four water levels is plotted against time of exposure. The points are means ± s.e.m., using the results from Tables 4.3 and 4.4.

FIGURE 4.18

DRY WEIGHT OF ORGANS (50 µg/l)

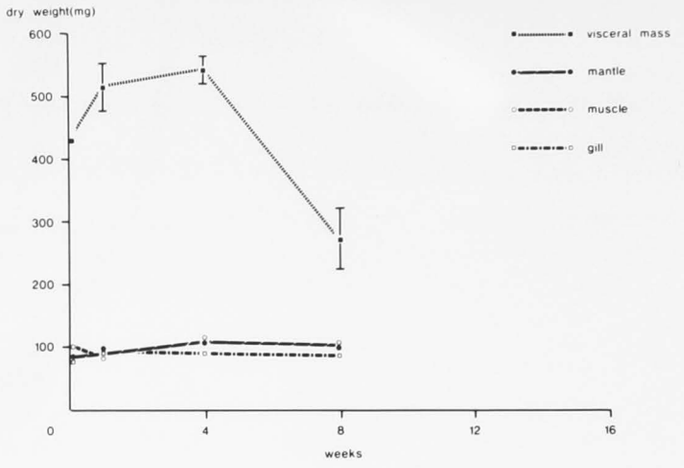


FIGURE 4.19

DRY WEIGHT OF ORGANS (150 µg/l)

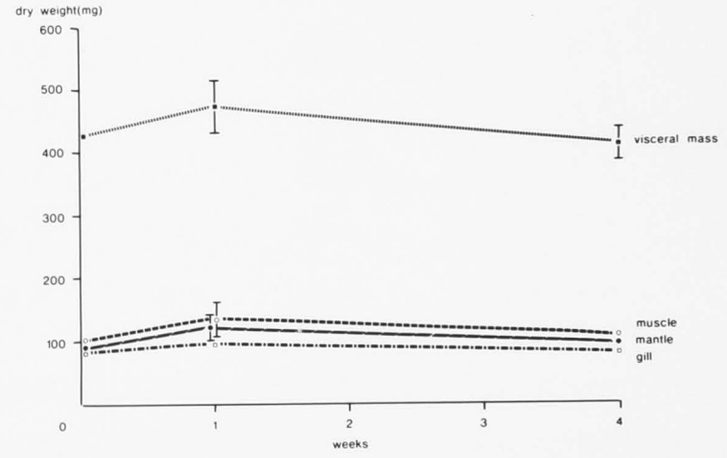


FIGURE 4.20

ACCUMULATION OF CADMIUM BY WHOLE OYSTERS

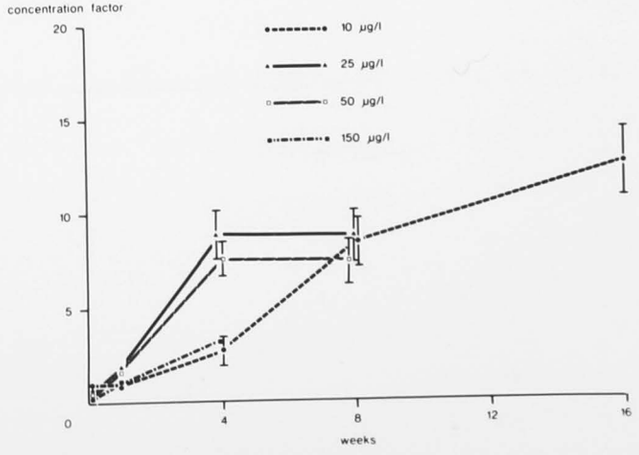


TABLE 4.5

Mean organ loads of cadmium as a % of the mean total body load.

Results from Tables 4.3 and 4.4 (- not assayed).

		V	Ma	G	Ms	H/K
Week 0		72.5	10.4	9.8	7.1	-
Week 1	0	59.6	17.1	14.6	9.0	-
	10	64.2	14.3	14.7	6.6	-
	25	71.8	7.8	15.6	4.7	-
	50	58.8	15.3	19.9	6.4	1.5
	150	52.9	14.1	18.9	10.6	3.6
Week 4	0	64.6	16.2	18.3	8.5	2.7
	10	58.6	10.6	18.1	7.4	5.2
	25	49.7	17.3	20.6	8.3	4.6
	50	48.7	15.1	21.8	8.8	5.5
	150	50.7	15.6	18.3	9.9	5.6
Week 8	0	64.0	13.6	11.7	10.9	-
	10	40.3	17.9	19.8	14.6	7.4
	25	56.7	9.8	15.7	11.4	6.5
	50	46.7	12.9	24.9	12.2	4.0

V = visceral mass

Ma = mantle

G = gill

Ms = muscle

H/K = heart/kidney

0 = control

10 = 10 µg/l

25 = 25 µg/l

50 = 50 µg/l

150 = 150 µg/l

the organs of those oysters. There are no significant differences (t test) between the week four and week eight concentrations of cadmium in the organs of oysters exposed to 25 and 50 µg/l cadmium. The plateau of the organ concentrations and the subsequent death of the animal may be causally related, but there is no direct proof to support that hypothesis. Therefore, as before, the significance of the appearance of the plateau of cadmium levels during accumulation is unknown.

The concentrations of cadmium reached by the gill are extremely high, 720 µg/g in the 150 µg/l group at week four, and 589 µg/g in the 50 µg/l group at week eight. O'Hara (1973) reports that fiddler crabs do not exceed 110 µg/g cadmium in gill tissue (presumably by wet weight), and postulates that the death of *Uca pugilator* in high cadmium levels is related to the accumulation of cadmium in the gills, although he also can present no direct proof.

For comparison, the results of the individual organ assays are plotted in Figures 4.3 to 4.7 as the cadmium concentration against time for each organ, and in Figures 4.9 to 4.12 as the concentration factor (cadmium level in organ/cadmium level in water) against time for each water concentration, except the controls. The results from the control group (Figure 4.8) are plotted as the concentration of cadmium against time, because the water level of cadmium is not known.

The greatest fraction of the total body load of cadmium is found in the visceral mass, followed by the gill and mantle, and then the muscle.

The general pattern of viscera > mantle > muscle > heart/

kidney is not altered by either level of cadmium treatment (up to 150 µg/l) or by time of exposure (up to eight weeks). However, the gills of oysters exposed to 50 µg/l cadmium contain 25% of the total body load of cadmium after eight weeks exposure, compared with 12% of body cadmium in the gills of the control oysters (Table 4.5). The two percentages are significantly different ($p < 0.05$, t test). This fact, together with the high concentration of cadmium achieved in the gill, indicates that the gill may be the critical organ for oyster survival during exposure to high cadmium levels. It is not possible to attribute directly the death of the oyster to the high cadmium load in the gill because the evidence, as discussed above, is circumstantial.

4.3.2.3 CHANGES IN ORGAN WEIGHTS

The mean dry weights of the organs at each exposure level (Table 4.4) are plotted against time in Figure 4.15 to 4.19.

In the 10 µg/l and control groups the visceral mass loses weight continuously up to week eight, and then stabilizes, while the other organs show no apparent weight losses up to 16 weeks. The 25 and 50 µg/l cadmium groups also show a loss of weight from the visceral mass, and no change in the weights of the other organs. The 150 µg/l group reaches 100% mortality between four and eight weeks, with none of the organs displaying any weight loss.

The loss of weight from the visceral mass in all groups probably indicates a partial starvation, with the oyster utilizing its reserves of glycogen in the visceral mass. The apparent retardation of the decline in weight in the viscera in the 50 and 150 µg/l groups may be due to an inhibition of glycogen degradation by cadmium.

Although the control oysters dropped from a mean dry weight of 699 mg at the beginning of the experiment to a mean dry weight of 518 mg at week 16, they appeared to remain in a "healthy" condition with the visceral mass and the mantle retaining glycogen. The effect of a decrease in condition on the accumulation of cadmium by the oysters in the experimental tanks is unknown.

Subjective observations of oysters from the 50 and 150 µg/l groups indicated that some organs, gill in particular, appeared to regress in the late stages of cadmium intoxication. The data on dry weights fails to support these observations.

4.3.3 AUTORADIOGRAPHY - RESULTS AND DISCUSSION

The pulse labelling technique is essentially a method for assessing the retention of recently accumulated cadmium. Organ loads that are turning over, or actively accumulating, will be seen as radioactive areas. Absence of radioactivity can be interpreted as lack of turnover or lack of organ accumulation at the time at which the radioactive pulse was provided. The specific activity of cadmium used as a pulse at each concentration was different. Therefore, there can be only comparison within concentrations and not between, since the amount of radioactivity in oysters from different concentrations is not a direct indication of the absolute amounts of cadmium absorbed. It is possible however, to assess the results on a time scale within each concentration.

The amount of ^{109}Cd retained in the oysters has been subjectively assessed on a scale of 1 to 4, where 1 is a detectable but very small quantity of label and 4 is a high average density of label (radioactive cadmium). The oysters were removed from the flowing

system at the same time as those for the assay of cadmium (4.2.1). The results are presented in table 4.6.

A high average density of label indicates that, at the time of sampling, a relatively large amount of cadmium was being absorbed and retained by the oyster. A low average density of label indicates that little cadmium was being absorbed and retained at the time of sampling. The technique does not assess the amount of cadmium being absorbed. If the cadmium in oyster tissues is in equilibrium with water cadmium, no retention will be observed. However, if the tissue cadmium and the water cadmium are in a steady state condition, or if the oyster is actively accumulating cadmium from the water, some retention of the labelled cadmium will be observed.

TABLE 4.6

The overall intensity of labelling with ^{109}Cd in autoradiographs. The scale of 1 to 4 indicates a subjective assessment of the average density of label over the whole autoradiograph.

	0 µg/l	10 µg/l	25 µg/l	50 µg/l	150 µg/l
Week 0	1	2	2	4	2
1	1	3	4	3	2
4	1	4	2	2	1
8	1	1	2	2	-

Some examples of the autoradiographs from which the results in Table 4.6 are drawn are shown in Plates 4.1, 4.2, 4.3 and 4.4.

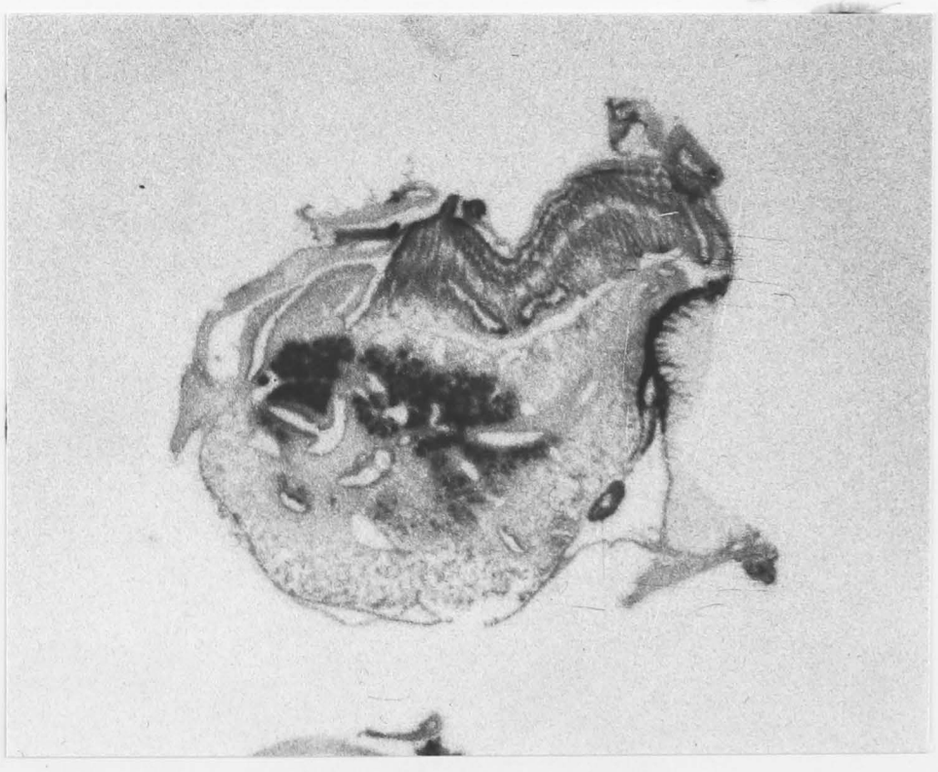
It seems that oysters exposed to lower concentrations of cadmium reach a peak of retention of cadmium at a later stage than those exposed to higher concentrations. At 25 µg/l cadmium maximum

PLATE 4.1

(a) Autoradiograph of a 100 µm section of an oyster. The oyster was exposed to 1 µCi/l ^{109}Cd and 10 µg/l $CdCl_2$ for four hours, followed by exposure to 10 µg/l $CdCl_2$ for 72 hours ("cold chase"). Sections were apposed directly to X-ray film, and the resulting silver deposits (black areas) represent areas of radioactivity in the oyster section.

(b) Autoradiograph of a section of oyster [as described in (a)]. The oyster was exposed to 10 µg/l cadmium for four weeks before exposing to radioactive cadmium.

a



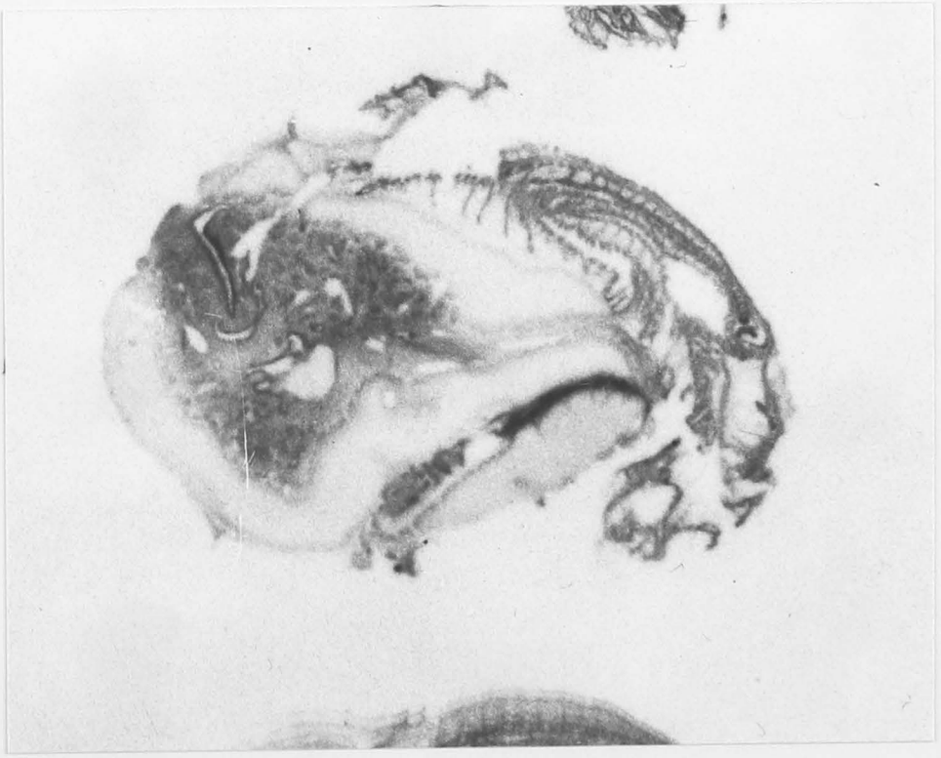
b

PLATE 4.2

(a) Autoradiograph as described in Plate 4.1, except that the concentration of $CdCl_2$ used was 50 µg/l. The oyster was not exposed to cadmium before the radioactive pulse of ^{109}Cd.

(b) Autoradiograph as described for (a), except that the oyster was exposed to 50 µg/l cadmium for one week before the radioactive pulse.

a



b

PLATE 4.3

(a) Autoradiograph as described in Plate 4.1, except that the concentration of cadmium used was 100 µg/l. The oyster was not exposed to cadmium before the radioactive pulse.

(b) Autoradiograph as described in (a), except that the oyster was exposed to 100 µg/l cadmium for four weeks before the radioactive pulse.

a

b

PLATE 4.4

(a) Autoradiograph as described in Plate 4.1, except that the concentration of cadmium used was 150 µg/l. The oyster was not exposed to cadmium before the radioactive pulse.

(b) Autoradiograph as described in (a), except that the oyster was exposed to 150 µg/l cadmium for one week before the radioactive pulse.

a

b

retention appears to be seen after one week's exposure to cadmium, while at background levels of cadmium very little cadmium is retained.

Figure 4.2 shows that the concentration of cadmium in the oysters exposed to 25 and 50 µg/l appears to have stabilized; however, Table 4.6 shows that (at week eight) some of the metal in the organs is exchanging with the water cadmium ("turning over"), even though the organs may have reached a plateau. This indicates a steady state condition between the oysters and the water at these two levels of cadmium. Also, it indicates that a particular level of cadmium *per se* is not directly responsible for death of the oyster since the animals die after week eight, although the level of cadmium has substantially plateaued at week four.

Unlike 150 µg/l, 25 and 50 µg/l cadmium may cause death indirectly, since a zone of resistance to cadmium is exhibited, while in the 150 µg/l group no such zone was observed.

4.3.4 GENERAL CONSIDERATIONS

The results indicate that oysters are capable of rapidly accumulating large amounts of cadmium, and reaching concentrations which are of substantial toxicity to both themselves and any consumers of their flesh. At a water concentration of 10 µg/l, the maximum recommended level of cadmium in drinking water (N.H. and M.R.C., 1972; and N.S.W. Clean Waters Act and Regulations, 1970), oysters can accumulate approximately 7.5 µg/g (dry weight) per week. A one week exposure to 10 µg/l cadmium (in the environmental conditions used for this experiment) is sufficient to cause oysters to accumulate enough cadmium to become unfit for human consumption (approximately 10 µg/g dry weight, assuming a background level of near 2 µg/g dry weight).

The consistency of the pattern of cadmium distribution between the organs (over both time and level of ambient cadmium) is striking and suggests a common mechanism for uptake at all levels and a common mechanism for storage, transfer and excretion of cadmium at all ambient levels.

It seems likely that accumulation of, and exposure to, cadmium has no effect on the dry weights of the organs of the oyster, with the possible exception of the visceral mass.

The concentration factors for whole oysters at the four concentrations examined in this experiment are shown in Figure 4.20. The points for the 10 µg/l and the 150 µg/l groups could be readily fitted to a straight line equation (although the latter group contained only three points). The 10 µg/l points can be described by $y = 0.76x + 0.69$ ($r = 0.98$, $p < 0.05$), and the 150 µg/l points by $y = 1.13x + 0.29$ ($r = 0.997$, $p = 0.05$). The 25 and 50 µg/l points cannot be fitted to a straight line. It is evident for cadmium in oysters that there is no consistent relation between concentration factors and time at each water concentration.

If there is no consistent relation between time and concentration factor for cadmium in oysters in the field, a knowledge of the concentration factors at any time cannot be used to predict past exposure to cadmium. Since the level of cadmium in oysters is not related in a consistent manner to water concentrations of cadmium (Figure 4.2) a knowledge of tissue levels of cadmium is of little use in predicting water concentrations of cadmium. Therefore natural populations of oysters may not be used as accurate indicators of cadmium concentrations in water over a period of time.

The factors affecting the extrapolation of the experimental results to predict the effects of cadmium in natural populations are:

time of immersion - the experimental oysters were continuously immersed (although they do not feed continuously) while natural populations spend only short periods feeding in a single 24 hr cycle;

temperature - the experimental oysters were maintained at a constant temperature (20 $\pm$ 2^{o}C) while natural populations are subjected to seasonal fluctuations (12 to 24^{o}C mean monthly temperature range near oyster leases in the Clyde River);

salinity - salinity in the experimental system was within the mean monthly range of salinities for water near an oyster lease in the Clyde River (25 to 33^{o}/oo);

food supply - since the gut is a site of excretion of cadmium the rate of passage of food may influence the excretion rates of cadmium, and hence the amount of cadmium accumulated;

constancy of toxicant level - the level of cadmium is unlikely to remain constant in the natural environment, but the effect of transient peaks of cadmium on the amount accumulated is unknown;

age - some evidence suggests a correlation between the level of metal and oyster age (e.g. Mackay, et al., 1975; Cunningham and Tripp, 1975b) while other work suggests that there is no correlation between age and level of metal (e.g. Ayling, 1974; Hugget, Bender and Slone, 1973). Thus, the effect of age on the reliability of the extrapolation from laboratory experiments to the field situation is unknown.

However, the qualitative assessment of cadmium water levels

using oyster cadmium concentrations may be a valid procedure. With a knowledge of the maximum values for cadmium in oysters from relatively unpolluted areas oyster tissue levels of cadmium could be screened to detect polluted waters. The probable long biological half life for cadmium in oysters may buffer the transient water peaks of cadmium, so tissue levels of cadmium may be used to indicate recent exposure to either pulses of cadmium or constant low levels of cadmium in water.

CHAPTER 5

THE LOCALIZATION AND ROUTE OF ACCUMULATION OF CADMIUM IN *C. COMMERCIALIS*

5.1 INTRODUCTION

Studies of the accumulation of metals by oysters rarely investigate, in detail, the actual sites at which they are bound. In this regard, the better studies concentrate upon the accumulation of metals in various organs of the animal, rather than in the whole animal (e.g. Segar, Collins and Riley, 1971). However, even at this, the organ level, they leave much to be desired, as few organs are composed of homogeneous masses of cells. Most organs are differentiated into specialized zones which are adapted to perform different functions. A thorough investigation of metal accumulation in any organism should continue beyond the organ level, to the cellular, the sub-cellular and even the molecular level. Such an approach has yielded at least some comprehension of the way in which vertebrates resist heavy metal challenge.

Brooks and Rumsby (1965) have summarized the possible routes of uptake of metals by marine animals and plants. The possible routes are, in general terms:

1. ingestion of particulate or food material containing metals;
2. complexing or exchange of metals with suitable surface ligands, with subsequent incorporation into physiological systems.

Cunningham and Tripp (1975a) briefly review recent evidence for metal uptake in bivalves. Their study of the accumulation and

distribution of mercury in *C. virginica* suggested that both the above routes contribute to whole body accumulation, although the gut appears to be the major site of absorption. The relative significance of the gill and the gut in accumulation depended upon whether mercury was presented in soluble or particulate form.

The gill had a greater residual mercury content when the mercury was presented as the soluble form, while the gut retained more when the challenge was via mercury-carrying algae. The ranking of accumulation in other organs was not altered by a change in the method of presentation (Cunningham and Tripp, 1975a). These workers also followed depuration of mercury after the two methods of exposure. The gill and the gut both lost considerable amounts of mercury which supports a role for them in accumulation, excretion, or subsequent transport to deeper tissues.

The precise mechanism by which metals are accumulated across the gills of bivalves is unknown (for example see Bryan, 1971). However, numerous studies have suggested that the gill is a direct accumulator of metal from the external environment (Galtsoff, 1964; Romeril, 1971; Cunningham and Tripp, 1975a). The gills of bivalves actively accumulate various nutrients from solution (see Chapter 7) and it is feasible that such active processes also accumulate metals, either directly or indirectly. However, Bryan (1971), discusses evidence for the possibility that metal accumulation in some aquatic organisms is a passive process, and that tissue concentrations of some metals reflect the water concentrations of those metals. A positive correlation between water levels and tissue levels suggests that the animal is unable to regulate its internal levels of the metal. Bryan

(1971) suggests that this may be the case for lead in *C. virginica*.

It seems possible that the uptake of zinc and copper (both essential trace elements) are also not regulated by oysters since much is freely removable from oyster tissues with little identifiable adverse effect (Wolfe, 1970b; Romeril, 1971; and Coombs, 1974). The results of Shuster and Pringle (1969) suggest that over a 20-week period the uptake of zinc, copper, cadmium and chromium is not regulated by *C. virginica*, although the correlation between the water levels and the tissue levels is indirect. Fowler and Benayoun (1974) and Schulz-Baldes (1974) found that, for zinc and lead accumulation respectively, tissue levels in *Mytilus* were proportional to water levels. The available evidence suggests, then, that the accumulation of cadmium by *C. commercialis* is likely to be a passive rather than an active process.

It has been proposed that the gut is the major route of accumulation of metals in *Mytilus edulis* (Pentreath, 1973), *Ostrea edulis* (Preston and Jefferies, 1969, cited in Bryan, 1971), *Ostrea sinuata*, *Mytilus edulis aoteanus*, *Pecten nova-zelandiae* (Brooks and Rumsby, 1965) and *C. virginica* (Cunningham and Tripp, 1975a; Preston, 1971, cited in Cunningham and Tripp, 1975a). The probable mechanism of accumulation is ingestion of food particles containing either absorbed or adsorbed metals. Oysters may ingest a great variety of organic and inorganic materials, including colloidal clay particles, bacteria, plankton and detritus particles in the 1 to 10 μm size range. Sequestering of ionic and colloidal metals by mucus is likely also to provide a further source of metals for the gut. Oysters trap and transport food-sized particles in mucus in the palps, and copious

amounts of mucus are used in the rejection of unwanted materials by the gill (forming pseudofaeces). The quantitative significance of mucus in trapping metals is unknown in oysters, both in its contribution of metals to the gut and in its capacity to transfer metals to the detritus food chain through the pseudofaeces.

The approach adopted, in Chapter 4, of assessing accumulation of cadmium organ by organ by atomic absorption spectroscopy is clearly limited when considering the cellular and sub-cellular sites of localization. In this chapter I attempt to consider the distribution of cadmium, organ by organ. Whole body autoradiographic techniques reveal the finer details of organ accumulation not discussed in Chapter 4. I also attempt to determine the route of accumulation, whether by gut or by gill and mantle and, finally, to examine the sub-cellular distribution of cadmium in the gill.

5.2 METHODS

5.2.1 LOCALIZATION OF CADMIUM IN ORGANS USING AUTORADIOGRAPHY - METHOD

The autoradiographic method is described in Chapter 4, and some of the results to be described in this chapter have been derived from autoradiographs prepared specifically for the experiments reported there. Other experiments also utilized the autoradiographic procedure, and this section is essentially a summary of all autoradiographic patterns seen throughout the entire study.

A standard technique for printing the autoradiographs was devised and all autoradiographs have been reproduced by the same photographic technique.

5.2.2 THE ACCUMULATION OF CADMIUM FROM *E. COLI* - METHOD

The culture and labelling of *E. coli* is described in detail in Chapter 6. 2.5 µCi ^{109}Cd (Amersham, "carrier free") was used instead of ^{14}C.

Two experiments were conducted in which oysters previously exposed to cadmium (0, 10 and 50 µg/l) in the flowing system for 12 weeks were fed on the suspension of *E. coli* labelled with ^{109}Cd. Three oysters per tank (500 ml) were exposed for 24 hours to sea water (no added $CdCl_2$) and labelled *E. coli* (15,500 cpm/500ml). Animals were shucked, rinsed three times in sea water, and then divided into visceral mass and the "rest". The heart/kidney remained with the "rest". A separate assessment of the visceral mass was undertaken to avoid the possible source of error of temporary gut retention of labelled bacteria. This may not, however, have been a significant problem since subsequent observations with carmine particles revealed a gut passage time of approximately three hours. Some body fluids may have been lost in the rinsing process, but it was considered more important to remove pseudofaeces clinging to gill and mantle. Tissue was added to tared counting vials and weighed, then an appropriate amount of NCS Tissue Solubilizer (Amersham/Searle) (four times the wet weight of tissue) was added to the vial and incubated for three days at 50^{o}C. One ml of the digest was added to one ml of a neutralizing solution (34 ml glacial acetic acid per litre distilled water) and mixed. Ten ml of PCS scintillant (Amersham/Searle) was added to the mixture, and allowed to stand at 4^{o}C in the dark for three days. The latter procedure reduced chemiluminescence to nil.

Water samples (4 ml) were centrifuged at 2300 G for 15 min at

4°C, and 2 ml of the supernatant was added to a counting vial with 2 ml of sea water and 10 ml of PCS scintillant. The pellet was resuspended and transferred to a counting vial, and 2 ml sea water was used to rinse the centrifuge tube and added to the counting vial, followed by scintillant. The two water fractions thus sampled are the particulate fraction (composed mostly of *E. coli*) and a "soluble" fraction of non-centrifugable material (probably mostly ionic and molecular species of cadmium).

Results are reported as cpm above background/g wet weight of oyster tissue, and as a percentage of the total counts available in each experimental tank (since these varied slightly from tank to tank). The results from the two experiments have been pooled.

5.2.3 ACCUMULATION OF CADMIUM FROM A SOLUBLE AND A PARTICULATE SOURCE - METHOD

Five experimental tanks were prepared with three "sterilized" oysters per tank, and one tank without oysters as a control. The oysters were previously unexposed to cadmium, and were "sterilized" according to the following schedule:

1. shells scrubbed with a nylon bristled brush, rinsed in tap water;
2. soaked in detergent/distilled water solution for 12 hours;
3. soaked in 0.1N HCl for one hour, followed by another scrubbing and three rinses in distilled water;
4. exposed for 12 hours to sterile sea water under ultra violet irradiation in a sterile room;
5. transferred in a sterile manner to the experimental tanks.

The experimental protocol is outlined below:

Tank number:	1	2	3	4	5	6
	ssw	ssw	ssw	ssw	ssw	ssw
	^{109}Cd coli	^{109}Cd coli	^{109}Cd(t)	^{109}Cd(s)	^{109}Cd(s)	^{109}Cd(t)
	oysters	-	oysters	oysters	oysters	oysters
	-	-	-	-	*E. coli*	*E. coli*

ssw = 500 ml of sterile sea water (0.22 µm filtered, autoclaved)

^{109}Cd coli = 10 ml *E. coli* suspension grown as before on ^{109}Cd.

^{109}Cd(t) = $^{109}CdCl_2$, 70 µl 0.66µCi/ml (3.4 µCi/mg Cd) stock solution.

^{109}Cd(s) = $^{109}CdCl_2$, 25 µl of the above stock solution.

E. coli = *E. coli* grown and fixed as above except that the cadmium is not added to the culture medium (unlabelled *E. coli*).

Oysters were allowed to feed undisturbed for 12 hours in the above system (at approximately 20^{o}C), and then in clean unfiltered sea water for a further 12 hours prior to analysis. Four organs (gill, mantle, muscle, and visceral mass) were removed and counted as before.

Water samples were taken (as in 5.2.2) from the tanks at the beginning and end of the experiment.

5.2.4 THE EARLY UPTAKE OF CADMIUM - METHOD

Oysters which had not been previously exposed to cadmium were "sterilized", then exposed, in individual tanks, to 0.415 µCi ^{109}Cd, and 50 µg/l $CdCl_2$ in 500 ml sea water. Each oyster was carefully monitored and after a fixed period of feeding the oyster was removed, shucked and immediately frozen in carbon dioxide in preparation for autoradiography as previously described. One oyster was frozen after each of 15 min, 30 min, 1 hr, 3 hr, and 24 hr of feeding.

5.2.5 THE SUB-CELLULAR DISTRIBUTION OF CADMIUM IN THE GILL - METHOD

Two experiments were conducted in which the sub-cellular distribution of cadmium was assessed in gill tissue. Three groups of oysters were used, first, oysters not previously exposed to cadmium, second, oysters exposed to cadmium in the flowing system (50 µg/l for 10 weeks), and third, oysters from the control tank of the flowing system (10 weeks, no added cadmium). The three groups were exposed to ^{109}Cd (0.45 µCi/oyster/500 ml) in unfiltered sea water for 24 hr, and then allowed to feed in clean unfiltered sea water for 48 hr. The gill was removed and homogenized with filtered sea water (8 ml) and stored on ice.

The homogenate was split into two parts (A and B) with both fractions being identical except that the mucous plug from the homogenate was added to fraction A. Fraction A was used to determine the amount of cadmium in the membrane fraction, the soluble fraction, and the < 5,000 and > 50,000 daltons molecular weight components of the soluble fraction. Fraction B was used to determine cadmium in the low speed membrane fraction, a "mitochondrial" fraction, and a "microsomal" fraction. The preparative technique was as follows:

A.	membrane fraction	107,000 G pellet (30 min)
	soluble	107,000 G supernatant (sn)
	< 5,000 m.w.	107,000 G sn passed through a Diaflo ultra-filtration cone
	>50,000 m.w.	soluble - < 5,000 m.w. fraction
B.	low speed membrane fraction	1,500 G pellet (10 min)
	mitochondrial fraction	15,000 G pellet (10 min)
	microsomal fraction	107,000 G pellet (30 min)

The "mitochondrial" fraction prepared in the above manner was bubbled for 10 min with nitrogen to remove oxygen from solution. 0.5 ml of 0.1 M succinate and two drops of 0.1% methylene blue were added to the deoxygenated solution. The sealed mixture decolourized overnight at room temperature, whilst a "microsomal" fraction treated identically did not decolourize. Methylene blue substitutes for oxygen as a terminal electron acceptor in mitochondrial oxidative phosphorylation, and becomes decolourized in the process. Decolourization may, therefore, indicate the presence of mitochondria in the fraction.

Samples were counted as previously described, using NCS Tissue Solubilizer for tissue samples and direct scintillation for aqueous samples in PCS scintillant.

5.2.6 THE LOCALIZATION OF HEAVY METALS IN THE GILL USING ELECTRON MICROSCOPY - METHOD

Sections of gill, approximately 1 cm. were quickly excised and fixed for 15 to 60 min in a 2½% glutaraldehyde, 2% NaCl, 0.1M Sorensens phosphate buffer (pH 7.4) solution. The tissue was then rinsed in buffer, and exposed to a 1% (w/v) ammonium sulphide solution until the tissue pieces turned dark brown (10 to 15 min). Tissue pieces were then washed in distilled water, and finally returned to the formaldehyde fixative overnight.

The following morning the tissues were washed in distilled water and processed under yellow safe lights in Pihl's (1967) modified Timm's developer (Table 5.1) for 30 to 60 min. The sections were then washed in three changes of distilled water, dehydrated in ascending concentrations of alcohol, transferred to propylene oxide and embedded

in Araldite. The blocks of tissue were cut on an ultra-microtome to exhibit gold interference colours and mounted on colloidin/carbon coated 200 mesh copper grids. The instrument used was a Phillips EM301 electron microscope.

Controls for the tissue treatment process were, first, tissue exposed to ammonium sulphide, but not developer, and second, tissue not exposed to ammonium sulphide but exposed to the developer.

The theory behind the technique is that the lightly fixed tissue precipitates sulphides at the site of heavy metal localization. The sulphide deposits are too small to be recognized, so the quinone developer precipitates a large, and electron dense, deposit at the sites of sulphide deposit. Low concentrations of heavy metals have been detected in mammalian cells using this (or a similar) method by Pihl (1967), Pihl and Falkmer (1967), and Haug (1967). The theory is discussed by Schmidt and Schultka (1974) and Popham and Webster (1976).

The gill pieces taken for examination were, in all cases, sampled from the central region of the gill.

TABLE 5.1

Timm's developer as modified by Pihl (1967)

30% (w/v) gum arabic 10% (w/v) sucrose	20 ml
hydroquinone	34 mg
10% citric acid - to adjust pH to 3.9 to 4.0	5-10 drops
0.1% silver nitrate	0.18 ml

The gum arabic/sucrose solution lasts about one month.

5.3 RESULTS AND DISCUSSION

5.3.1 LOCALIZATION OF CADMIUM BY AUTORADIOGRAPHY

The whole body autoradiographic technique is of particular value in assessing relative densities of metal accumulation within or between organs. The visceral mass is of special interest since in Chapter 4 it was treated as a single organ; however, it has such components as the gut and its associated digestive diverticulae, the gonad, and areas of considerable glycogen storage. Similarly both the adductor muscle and the heart/kidney were treated as units in the earlier discussion. The autoradiographs reveal more about the internal distribution of metal binding.

Typical autoradiographs are presented in Plate 5.1. The sections of oyster from which they were prepared are shown, stained, in Plate 5.2. There are several areas of highly localized cadmium accumulation. They are, in probable order of significance, gut and digestive diverticulae, heart/kidney, gill and mantle. Other areas, while showing some radioactivity, are more minor accumulators. Radioactivity is very low in muscle (both types), gonad, and glycogen storage areas of the visceral mass. The mantle is usually observed in transverse section, and shows a variable degree of labelling.

The low level of labelling in the gonad and glycogen storage areas of the visceral mass is of dual significance. First, it may indicate little or no effect on either gonad development or function, or on glycogen storage and mobilization. Second, since the gonad and the glycogen storage can amount to a very large fraction of the oysters' total wet weight (often 40 to 50%, depending upon the season), (Galtsoff, 1964), the lack of cadmium accumulation in these areas will

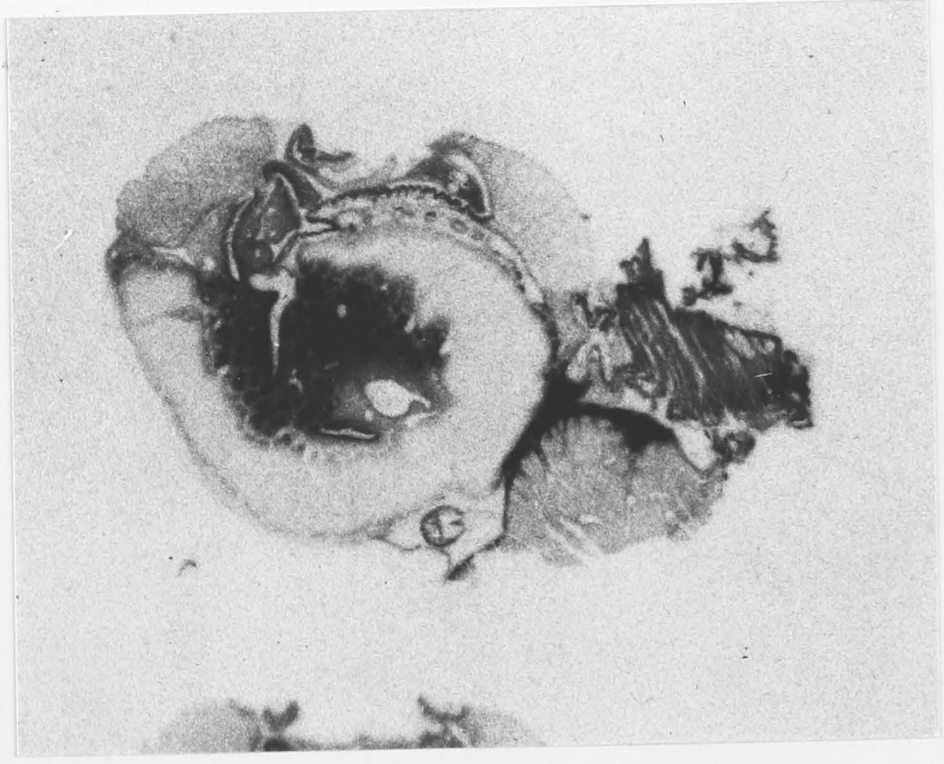
PLATE 5.1(a)

An autoradiograph of an oyster containing radioactive cadmium. The oyster was frozen in carbon dioxide and a 100 μm section exposed to X-ray film. The black areas on the autoradiograph represent radioactive regions in the oyster.

PLATE 5.1(b)

An autoradiograph, prepared as in (a), of a sexually mature oyster. Label in the area of the gonad is minimal.

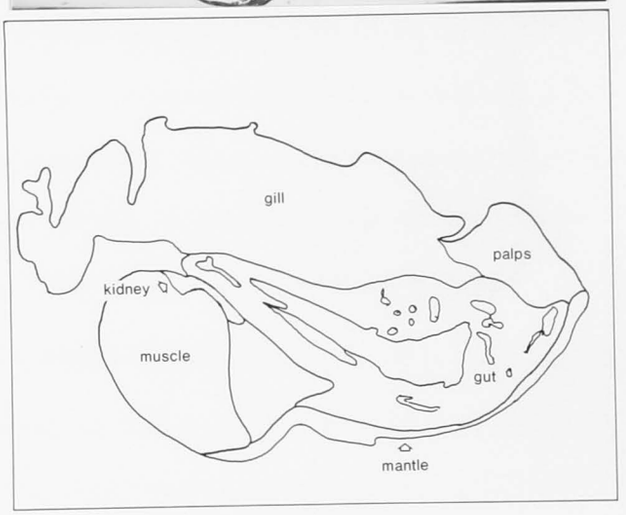
a

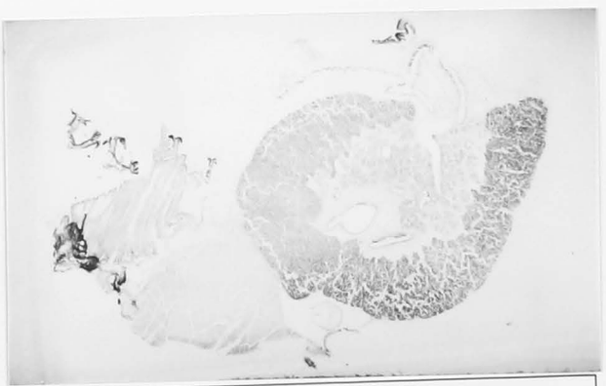


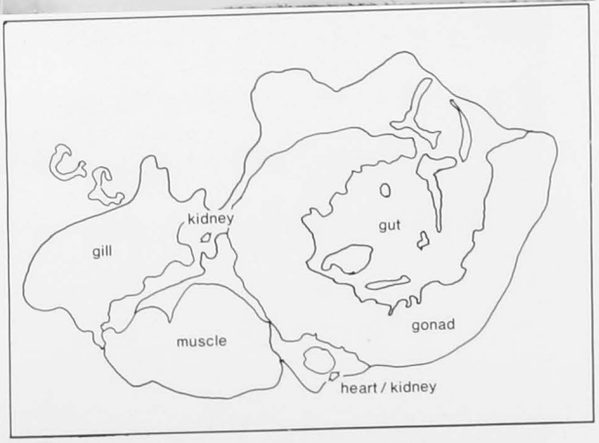
b

PLATE 5.2(a) and (b)

The fixed stained and mounted oyster sections used to prepare the autoradiographs in Plate 5.1.


gill
palps
kidney
muscle
gut
mantle




kidney
gut
gill
gonad
muscle
heart / kidney

cause an apparent seasonal fluctuation in cadmium concentrations. Although the concentration of cadmium in the whole oyster may change the absolute amount of cadmium in the oyster is unlikely to fluctuate markedly and the concentration of cadmium in the organs where cadmium is accumulated may not change at all.

Cunningham and Tripp (1973) report a decline in mercury tissue concentrations during the accumulation period in *C. virginica* exposed to mercuric acetate, attributed to spawning which occurred in both experimental groups. Unfortunately, they do not report the amount of metal (or the tissue weights), so it is not possible to ascertain that fraction of the total body load of metal lost at spawning.

Cunningham and Tripp (1975b) also observed a drop in mercury concentrations during accumulation of 100 µg/l mercury by *C. virginica*. The drop in concentration seen in that experiment is also attributed to the fact that the oysters had spawned, although a similar drop in metal concentration was not observed at a lower level (10 µg/l) or in the controls. The mean level of mercury in gonad during accumulation (approximately 10%, Cunningham and Tripp, 1975a) is adequate to explain a loss of metal, as proposed by Cunningham and Tripp (1975b). The distribution of cadmium among the tissues of *C. commercialis* makes it unlikely that spawning would reduce cadmium concentrations. Rather, it is possible that concentrations of cadmium in *C. commercialis* would increase because of loss of non-cadmium containing tissue.

Given the recognized seasonality of glycogen accumulation and gonad maturation, correlating changes in cadmium concentrations with seasonal or other effects could lead to erroneous conclusions if the absolute amount of the metal is not also considered. Spawning may

cause an apparent rise in the concentration of cadmium, if it is expressed in terms of body mass, without a change in the absolute amount of the metal present in oysters. Ruddell and Rains (1975) have independently arrived at a similar conclusion for zinc in oysters, using a simple mathematical assessment of apparent fluctuations of zinc concentrations in oysters.

The general pattern of cadmium distribution, apart from the gonad, is similar to that observed for other metals in oysters (zinc [Romerill, 1971]; mercury [Cunningham and Tripp, 1975a]), and to the pattern of cadmium distribution observed in *Ostrea sinuata* by Brooks and Rumsby (1967). However, the distribution of cadmium in other marine molluscs is variable (Segar, Collins and Riley (1971) and is not consistent with that reported here.

The sequence of events suggested by the autoradiographs indicates that the gill and mantle are probably capable of accumulating ionic or loosely complexed cadmium from ingested particulate matter, and perhaps from mucus with sequestered ionic or colloidal cadmium. The metal enters the blood-stream, since the heart/kidney is intensely labelled, and may be transported to the gill and mantle by the blood.

Cadmium accumulated in the gut may be transferred to the other organs by the wandering phagocytes. Phagocytes are closely associated with intra-cellular digestion (Tripp, 1963) and are capable of engulfing both particulate and dissolved material from the gut. Subsequently, they may transfer the metals to the blood, and they may be carried by the blood to the gill, mantle or other organs.

The sites of cadmium accumulation may be quantitatively influenced by the route of accumulation. If, for instance, the gill

and mantle are capable of accumulating large amounts of cadmium from the soluble phase of the water column, a challenge with soluble cadmium should cause a shift in emphasis from the gut as the major site of accumulation to the gill and mantle. Alternatively, if the cadmium was provided as particulate material the gill and mantle may play a more minor role in accumulation.

5.3.2 ACCUMULATION OF CADMIUM FROM *E. COLI*

The autoradiographic study of section 5.3.1 suggests that the incorporation of cadmium is via the gut of *C. commercialis*, and a series of experiments have been conducted to examine this route further. *Escherichia coli* were grown on $^{109}CdCl_2$ and a suspension of the bacteria in sea water was transferred to tanks in which oysters were feeding.

E. coli is approximately one µm in length (in its unaggregated state) and is therefore of a suitable size for ingestion by oysters. Other bacteria are ingested and digested by oysters (Cheng and Rudo, 1976). Evidence presented in Chapter 6 further supports the contention that *E. coli* is ingested and digested by oysters.

Oysters exposed to cadmium (control, 10 µg/l and 50 µg/l) in the flowing sea water system for 12 weeks were allowed to feed on ^{109}Cd labelled *E. coli* for 24 hours. The soft tissues of the oysters were then divided into two parts (the viscera and the "rest"), and weighed, solubilized and counted by scintillation. The results are presented in Table 5.2.

TABLE 5.2

The effect of cadmium pretreatment on the incorporation of cadmium by oysters from ^{109}Cd labelled *E. coli*.

Control		10 µg/l		50 µg/l	
Viscera	Rest	Viscera	Rest	Viscera	Rest
693	811	210	207	391	481
1765	669	1087	907	578	683
377	518	235	191	229	296
237	189	1191	1073	524	306
2091	688	279	196	211	179
313	644	1120	844	461	248

(a) ^{109}cadmium, cpm above background/g wet weight oyster tissue.

3.78	4.43	1.18	1.17	2.47	3.04
9.64	3.66	6.12	5.11	3.65	4.31
2.05	2.83	1.32	1.08	1.34	1.87
1.36	1.08	6.71	6.55	3.21	1.87
11.99	3.94	1.57	1.19	1.29	1.09
1.79	3.69	6.31	5.15	2.83	1.52
$\bar{x}$ 5.1	3.27	3.86	3.37	2.48	2.28

(b) ^{109}cadmium, cpm above background/g wet weight as a % of initial total available cpm/tank (results from (a)).

The loss of ^{109}cadmium from the water in each tank was followed, and the results are presented in Table 5.3.

TABLE 5.3

The loss of ^{109}cadmium from the water during feeding of oysters on ^{109}cadmium labelled *E. coli*.

The mean ^{109}Cd levels for all tanks at the start and finish of the experiment.

	Soluble (cpm/tank)	Particulate (cpm/tank)
start	3,437	17,000
finish	5,833	4,333

Total loss of cadmium = 10,271.

The loss of label from the particulate fraction suggests that the oysters remove the bacteria from suspension, and the appearance of ^{109}cadmium in the visceral mass and other organs indicates an accumulation of cadmium from the bacteria. There is no statistically significant effect of cadmium pretreatment on the level of ^{109}cadmium incorporated into the tissues.

The mean level of ^{109}cadmium accumulated per tank by the oysters is 587 cpm/g (total, 5,804 cpm), while the mean initial level of label in the water is 20,437 cpm, a removal or accumulation efficiency (of 1g tissue in 500 ml sea water) of 2.87%. Some label was found in a sample of faeces, and it is likely that cadmium in faeces and pseudofaeces, the loss of body fluids, and adsorption of cadmium on to oyster shells and the glass walls of the experimental tanks account for the approximately 5,000 cpm unaccounted for in this experiment.

Although the fixed *E. coli* were washed twice in sea water (4^{o}C) after preparation, a considerable amount of ^{109}Cd (mean 20%) was associated with the soluble fraction in the experimental tanks. It is

possible that the oysters remove the ^{109}Cd from this soluble fraction, and not from the bacteria or insoluble fraction. This would be possible if the two pools of cadmium (particulate and soluble) were in a rapidly reached steady state. Irrespective of this problem, approximately 50% of the total ^{109}Cd accumulated was found in the visceral mass, indicating a major role for the gut in accumulation.

5.3.2.1 THE LABILITY OF *E. COLI* BOUND ^{109}CADMIUM

An experiment was conducted to examine further the possibility that the oysters remove ^{109}cadmium solely from sea water, and not from the ^{109}cadmium labelled *E. coli*. It involved an assessment of the lability of ^{109}cadmium bound to *E. coli*.

The bacteria were grown and processed as before. An aliquot (10 ml) of the final suspension was placed in a dialysis tube and dialyzed for 24 hours at 20°C against sea water (500 ml). After the 24 hr the dialysis tube was shaken to mix the contents thoroughly, an aliquot was removed, centrifuged, and counted. The sea water was also sampled and counted although not centrifuged. The results are outlined below:

	Start of Experiment	Finish of Experiment
^{109}cadmium in *E. coli* (total cpm)	13,102	6,650
Supernatant in dialysis tube (total cpm)	907 (90.7 cpm/ml)	600 (60.0 cpm/ml)
Ratio $\frac{\text{total cpm } E. coli}{\text{total cpm supernatant in dialysis tube}}$	6.5%	8.2%
^{109}Cd in sea water (total cpm)	100	5,625 (11.25 cpm/ml)

Despite a loss of approximately 40% of the total counts to

the sea water, the ratio of soluble to particulate fractions remains near 7%, and the counts in the particulate fraction decrease considerably. Approximately 25% of the total label available is incorporated by oysters over a 24-hour exposure to labelled *E. coli* (5.3.2). It would therefore appear that accumulation may be explained by adsorption of cadmium by oysters from the soluble phase rather than the particulate fraction.

Oysters remove most *E. coli* from suspension in about ten hours of feeding time. If the oysters in the experiments discussed above were removing cadmium solely from the soluble phase after about ten hours the level of label in the soluble phase would begin to decrease (when the bacterial store of cadmium has been depleted). Instead, the level of cadmium rises slightly in the soluble fraction, and falls to about 25% of its initial level in the particulate fraction. This is circumstantial evidence for the direct removal by the oysters of much *E. coli* from suspension.* However, since some soluble cadmium is present in the experimental system, the results in Tables 5.2 and 5.3 may represent the sum of both routes of accumulation (from the bacteria and from the water), and be complicated to a small extent by a progressive loss of cadmium from the bacteria to the soluble phase of the water column.

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* Feeding experiments in Chapter 6 are not subjected to a similar scrutiny of the soluble/particulate ratios because the label there is ^{14}C, the soluble/particulate ratio is low and constant, and some direct observations of the water samples indicate the rapid removal of bacteria from suspension. A conclusion from the work in Chapter 6 is that oysters rapidly remove bacteria directly from suspension, and since the experimental conditions are similar to those in the experiments of the present chapter, a similar conclusion can be drawn here.

5.3.3 ACCUMULATION OF CADMIUM FROM A SOLUBLE AND A PARTICULATE SOURCE

The relative abilities of the major organs of the oyster to accumulate cadmium from both soluble and particulate sources is investigated in this experiment.

Because of the difficulty of preparing a particulate form of cadmium free of soluble cadmium, the two challenges (soluble and particulate) used in the experiment are not purely of one type, but rather with one type predominating over the other. It seemed possible that the organ accumulation would reflect the different types of challenge.

Oysters were exposed to ^{109}cadmium labelled *E. coli* (Tank 1), ^{109}cadmium chloride at two levels (Tanks 3 and 4), and ^{109}cadmium chloride at two levels with added unlabelled *E. coli* (Tanks 5 and 6). Tank 2 is the control tank.

The results are expressed as cpm/g tissue, and as a % of the total initial cpm available in each experimental tank in Table 5.4(a). The mean level of accumulation of ^{109}cadmium by whole oysters is shown in Table 5.4(b).

TABLE 5.4 (a)

Organ levels of ^{109}Cd, expressed as cpm above background/g wet wt tissue for each organ, and as a % of the initial total cpm/tank.

Tank	Gill		Mantle		Muscle		Visceral Mass	
	cpm/g	%	cpm/g	%	cpm/g	%	cpm/g	%
1	1632	1.77	1224	1.33	768	0.83	1604	1.74
(^{109}Cd	1888	2.05	1044	1.13	536	0.58	1415	1.53
E. coli)	1680	1.82	506	0.55	258	0.28	526	0.57
	$\bar{x}$ 1.88		1.00		0.56		1.28	
2	CONTROL TANK - NO OYSTERS							
3	3378	3.43	1426	1.44	636	0.64	1866	1.89
($^{109}CdCl_2$)	1180	1.20	672	0.68	516	0.52	931	0.94
	1510	1.53	932	0.94	638	0.65	1397	1.42
	$\bar{x}$ 2.05		1.02		0.60		1.42	
4	626	1.90	375	1.14	291	0.88	382	1.16
($^{109}CdCl2$)	1183	3.60	344	1.04	182	0.55	593	1.80
	1126	3.43	658	2.0	578	2.00	834	2.54
	$\bar{x}$ 2.97		1.39		1.14		1.83	
5	596	1.59	392	1.05	200	0.54	432	1.16
($^{109}CdCl_2$	752	2.01	528	1.41	227	0.61	599	1.60
+ *E. coli*)	713	1.91	550	1.47	488	1.30	738	1.97
	$\bar{x}$ 1.84		1.31		0.82		1.58	
6	1827	1.81	1466	1.44	973	0.96	2043	2.02
($^{109}CdCl_2$	1056	1.04	1096	1.08	851	0.84	540	0.53
+ *E. coli*)	1870	1.85	1955	1.93	580	0.57	1882	1.86
	$\bar{x}$ 1.57		1.48		0.79		1.47	

Anova table (percentages)		df	ms	F
	columns	3	4.28	13.77*
	rows	4	0.78	2.50
* $P < 0.05$	interaction	12	0.17	0.56
	error	40	0.31	

TABLE 5.4(b)

Mean accumulation of ^{109}Cd per oyster (cpm/g wet wt) as % of the initial available cpm ^{109}Cd (results from (a)).

Tank 1	2	3	4	5	6
4.73	-	5.09	7.35	5.54	5.31

(no significant differences by t test)

The level of ^{109}cadmium in both soluble and particulate fractions of the water column was assessed at the beginning and end of the experiment. The results are presented in Table 5.5. The total ^{109}cadmium per tank at each time is presented, but, only the ratio of the insoluble/soluble ^{109}cadmium is shown.

TABLE 5.5

Water levels of ^{109}Cd (cpm above background).

Tank	1	2	3	4	5	6
0 hr cpm (total)	92250	70000	98625	32875	37375	101125
12 hr cpm (total)	53375	74250	42375	12625	15875	48250
% loss of ^{109}Cd from water between 0 hr and 12 hr	42.1	-	57.0	61.6	57.5	52.3
0 hr ratio insol/sol	0.970	0.650	< .001	.065	.081	.002
12 hr ratio insol/sol	0.10	0.131	0.065	0.166	0.075	0.196

From the results in Table 5.5 it is obvious that, although the ^{109}cadmium was added to tanks 3, 4, 5 and 6 as soluble $CdCl_2$ (or Cd^{2+}), initially a small % of the total cadmium present (up to 8% in tank 5) was present in an "insoluble" form (capable of being centrifuged out of suspension by 2300 G). In tanks 5 and 6 this is probably due to the adsorption of cadmium by the unlabelled *E. coli*.

After the 12 hour experimental period the fraction capable of being centrifuged out of suspension increased to nearly 20% of the total (tank 6). However, the insoluble fraction in tanks 3 and 4 also increased slightly over the 12 hr period. The increase in the latter two tanks may be explained by:

1. release from the oysters of bacteria or other particulate material retained by them during the "sterilization" process, and subsequent adsorption of cadmium;
2. Growth of air borne bacteria in the sea water, and subsequent absorption of cadmium; and
3. the formation of large cadmium colloids or other complexes.

The presentation of cadmium in different forms has no significant effect on the cadmium accumulated by the organs (Table 5.4). Therefore, despite the uncertainties about the actual form of dissolved cadmium, and the loss of cadmium from bacteria to solution, the presentation of cadmium in two dissimilar forms has no effect on either total ^{109}cadmium accumulation or the organ distribution of ^{109}cadmium. Also, the presence of food size particles (unlabelled *E. coli*) has no significant effect on either the accumulation or organ distribution of labelled cadmium.

The experimental results show that, after a 12 hr exposure to ^{109}cadmium and a subsequent 12 hr period of depuration, the relative magnitude of cadmium binding in the organs is gill > visceral mass > mantle > muscle. The ranking of the organs is not affected by the forms of challenge (i.e. soluble or particulate).

There are at least two possible mechanisms of accumulation which would lead to the preceding conclusions. First, a continuous ingestion of mucus would provide a source of metals for the gut

irrespective of the form of cadmium, since it is possible that the mucus can scavenge soluble, colloidal and particulate metal from the water column. Second, the intake of cadmium may be mainly across the gill and mantle, from which it is transported to the gut in the blood. Phagocytes are transported around the body in the blood and may excrete cadmium across the epithelial lining of the gut, to be voided with the faeces. In the latter case the gut would be acting as an organ of excretion rather than accumulation. The two possibilities are not mutually exclusive, and may operate simultaneously. Neither possibility is satisfactory to explain the predominance of the gill as the major accumulating organ.

5.3.4 EARLY UPTAKE

An experiment in which autoradiography was used was performed to examine the time course of uptake of soluble cadmium. It was reasoned that such a study might reveal the translocation of metals from organ to organ, in the very early stages of accumulation, and might be used to interpret further the results of 5.3.3.

The results, in the form of autoradiographs, are shown in Plates 5.3 to 5.6. They show that, at 15 min there is an initial trace of label over both the gill and gut, which is likely to be background contamination from the labelled sea water. After one hr the gut and the heart/kidney appear to be more strongly labelled than the gill. At 24 hr the gut is clearly the most strongly labelled organ, closely followed by the heart/kidney. The appearance of label in the heart/kidney seems to parallel that of the gut, indicating transport of cadmium through the gut into the blood, with subsequent transport to the heart/kidney. In this process the gut appears to retain much

PLATE 5.3

Autoradiograph, prepared by freezing an oyster in CO_2, sectioning and then apposing to X-ray film. The oyster was exposed to radioactive cadmium (0.415 µCi) for 15 min before being frozen.

PLATE 5.4

Autoradiograph, prepared as in Plate 5.3. The oyster was exposed to radioactive cadmium (0.415 µCi) for one hour.

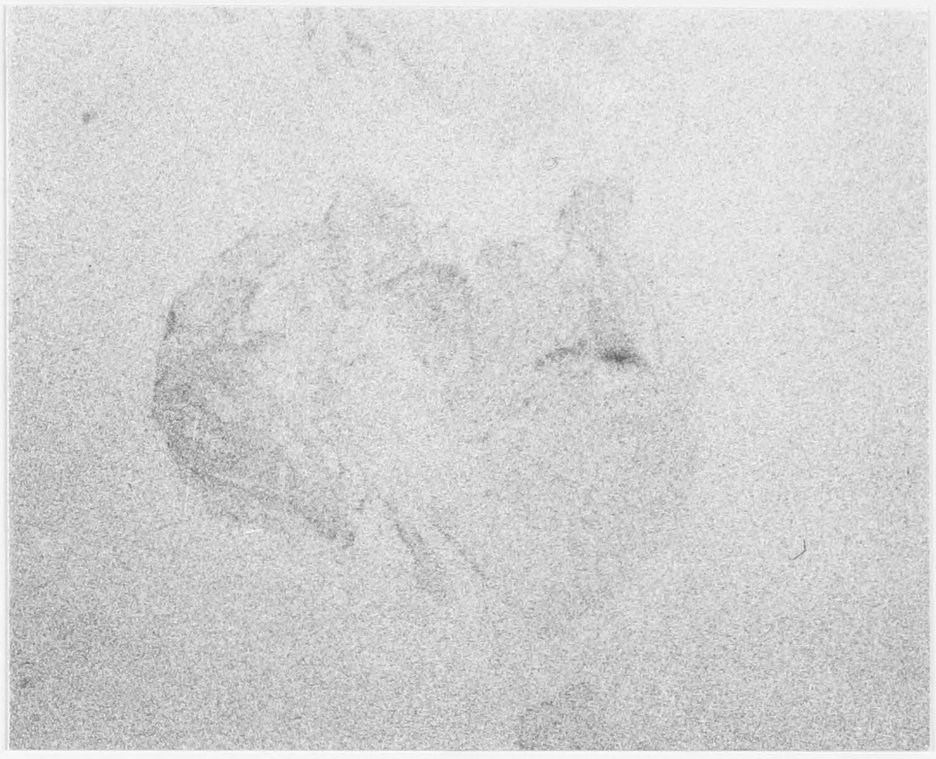
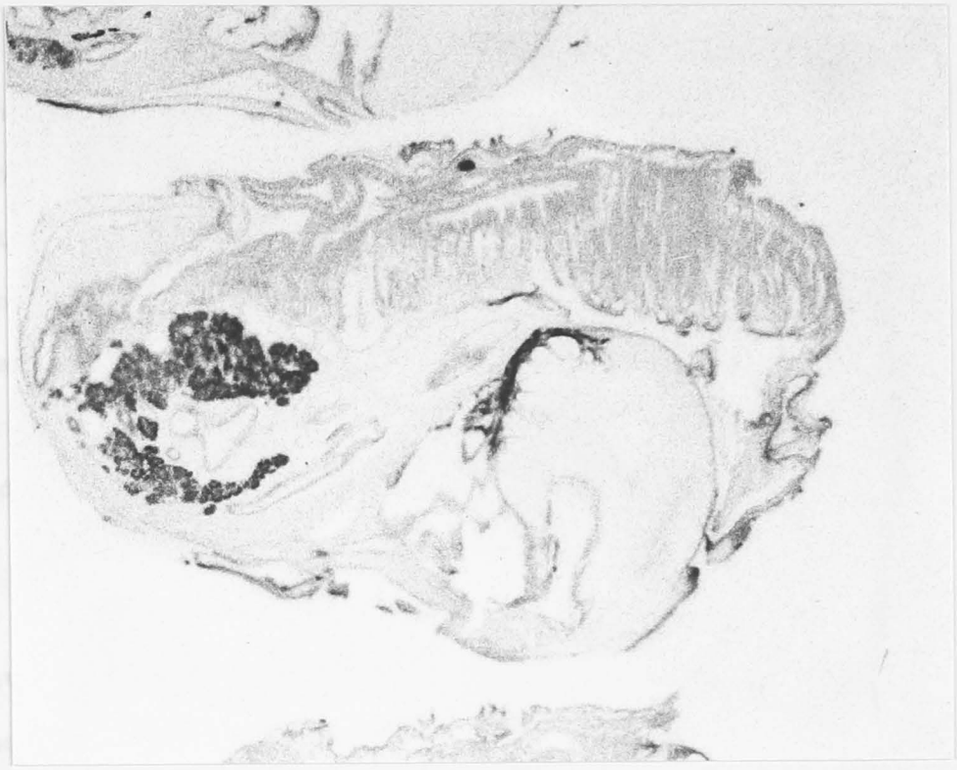


PLATE 5.5

Autoradiograph, prepared as in Plate 5.3. The oyster has been exposed to radioactive cadmium (0.415 µCi) for three hours.

PLATE 5.6

Autoradiograph, prepared as in Plate 5.3. The oyster was exposed to radioactive cadmium (0.415 µCi) for 24 hours.



cadmium in the digestive diverticulae. The alternative mechanism is, as described in 5.3.3, that the metal is absorbed across the gill and mantle into the blood, and subsequently transported to the gut and heart/kidney for excretion. I favour the first alternative, that the metal is absorbed mainly through the gut into the blood, since it is difficult to envisage the gill transporting a large fraction of the total load of cadmium across the cells of the epithelium without retaining much of that metal. The level of cadmium in the gill continues to rise for many weeks during exposure to cadmium at this concentration (Chapter 4), and so the binding sites for cadmium are clearly not saturated at 24 hr. Takatsuki (1934), Stauber (1950) and Tripp (1960) show that few phagocytes (amoebocytes) cross the gill epithelium, and so it is unlikely that cadmium can be absorbed directly into these cells at the gill surface, and hence into the blood.

If the gut is labelled directly from the external medium (in a soluble challenge), rather than from the translocation of cadmium from other organs, it is important to consider the possible mechanisms. In 5.3.3 the mucus was suggested as a possible mechanism and, since drinking of water has not been reported in oysters, the mucus seems to be the most probable source of cadmium for the gut. The later views reported in the literature suggest that mucus is used only selectively by oysters in feeding (for example, Owen, 1966), but it is not clear whether the palps and gill continue to produce mucus for ingestion even in the absence of food size particles. The drop in pH in the gut (Galtsoff, 1964) would be of great assistance in liberating the metals adsorbed or complexed with mucus, and it is unlikely that mucus is passed through the gut without some other form of digestive degradation,

which would also aid in the release of metals.

Although cadmium may not traverse the epithelial cells of the gill to the blood, it is likely that the epithelial cells directly accumulate cadmium from the water. The level of intensity of label in the gill does not appear to increase as rapidly as that in the gut and the heart/kidney, so a different mechanism - direct accumulation from water - may be operating.

5.3.5 THE SUB-CELLULAR DISTRIBUTION OF CADMIUM IN THE GILL

The widespread presence in vertebrates of a protein which binds heavy metals with great avidity (metallothionein) has prompted a search for analogous proteins in other animals. Coombs (1974) concludes that neither metallothionein nor any similar protein for binding zinc and copper exists in oysters. Zinc binding in oysters is different from that in vertebrates (Wolf, 1970; Romeril, 1971; Coombs, 1974; Friberg, et al., 1974) although the molecular characteristics of cadmium binding in oysters are unknown.

In previous studies on metal binding in oysters either ultrafiltration (Coombs, 1974) or centrifugation (Romeril, 1971) has been used to examine the distribution of metals between the different sub-cellular fractions.

Despite the disadvantages, I have chosen the centrifugation technique to examine the distribution of cadmium in the sub-cellular fractions of the oyster gill. The effects of homogenization and the redistribution of metal between compartments during preparation, and the effects of pressure in the centrifugation system are all unknown.

Oysters pretreated with cadmium for ten weeks were exposed to

^{109}cadmium for 24 hr and then to fresh sea water for 48 hr. The gills were removed, homogenized and split into two fractions (A and B). Fraction A was used to determine membrane bound and soluble ^{109}cadmium, and Fraction B to further partition the membrane bound fraction of A.

The results are presented as cpm/whole gill as a fraction of the total counts (corrected for background). The two groups of oysters not treated with cadmium are treated as one group (control) for analysis. A value of 0.5 in the tables below indicates that one sub-cellular fraction contains half of the total ^{109}cadmium absorbed.

Fraction A -

	Control						$\bar{x}$	50 µg/l					$\bar{x}$
membrane bound ^{109}Cd	.47	.44	.34	.48	.44	.49	.44	.40	.51	.57	.53	.55	.51
soluble ^{109}Cd	.53	.56	.56	.65	.53	.51	.55	.60	.49	.43	.46	.45	.49

The soluble ^{109}Cd fraction is divided into two further components.

	Control						$\bar{x}$	50 µg/l					$\bar{x}$
< 5,000	.30	.31	.22	.61	.65	.29	.40	.31	.17	.14	.15	.17	.19*
> 50,000	.24	.25	.34	.03	-	.22	.18	.29	.32	.29	.31	.28	.30

Fraction B - the membrane bound ^{109}Cd fraction was separated into three components:

	Control						$\bar{x}$	50 µg/l					$\bar{x}$
low speed membrane	.26	.25	.22	.15	.24	.21	.22	.24	.32	.29	.30	.27	.29*
mito-chondrial	.13	.10	.18	.03	.05	.05	.09	.08	.06	.05	.02	.03	.05
micro-somal	.05	.04	.08	.03	.01	.04	.04	.03	.04	.04	.04	.03	.03

* significantly different from control ($P < 0.05$, t test)

It is apparent that labelled cadmium is distributed evenly between the membrane and the soluble sub-cellular fractions, and that a long term exposure to cadmium does not alter that distribution. However, within the cytosol (soluble) component, more labelled cadmium seems to be associated with the low (< 5000) molecular weight fraction than the > 50000 m.w. fraction in untreated oysters. This situation is reversed by cadmium pretreatment, with the majority of the label then being associated with the higher molecular weight fraction. The mitochondrial and microsomal fractions contribute very little label to the membrane fraction, with most label being found in the low speed pellet. Pretreatment with cadmium enhances the amount of labelled cadmium associated with the low speed membrane fraction.

The low molecular weight component of the cytosol contains the largest single amount of label, in the oysters not pretreated with cadmium. The reversal of this situation in pretreated oysters may indicate a saturation of low molecular weight ligands by cadmium from the prior exposure. The increase in labelled cadmium associated with

the low speed membrane fraction may also reflect saturation of the < 5000 m.w. binding sites.

The distribution of sub-cellular zinc (Romeril, 1971) differs considerably from that reported here for cadmium. In the oyster gill approximately 90% of zinc is found in the membrane fraction with approximately 10% in the cytosol, compared to approximately 50% cadmium in each fraction reported here.

Brooks and Rumsby (1967) report a cadmium level of 19.6% in the cell debris (membrane) fraction of cells from heart tissue, and that approximately 50% of the heart cadmium is found in a soluble non-dialyzable fraction (firmly bound to cytosol components). These results differ from those obtained by Romeril (1971) for zinc in oyster hearts, and again indicate that the sub-cellular distribution of cadmium may not parallel that of zinc in oysters.

After two washes with phosphate buffer approximately 20% of the cadmium is firmly associated with the membrane fraction of oyster hearts (Brooks and Rumsby, 1967). This is less than the percentage of cadmium, reported above, found in the membrane fraction of gill tissue (50%), and much less than the 80% of zinc associated with the oyster heart membrane fraction (Romeril, 1971). The technique of Brooks and Rumsby (1967) may have removed loosely associated cadmium from the membrane fraction of the heart homogenate (since phosphate buffer can readily complex with cadmium [Perrin and Dempsey, 1974]), but there may also be inter-organ differences which could explain the discrepancy between the two figures for cadmium.

The cytosol fraction of homogenates of whole oysters contains

approximately 40% of the total zinc and copper (Coombs, 1974), but a comparison with the present study is difficult because of the likely inter-organ differences between the levels of zinc (Romeril, 1971) and possibly copper.

The experiment described in this section considers gill homogenates and is therefore not able to discriminate between the different cell types of the gill. The following section (5.3.6) attempts to assess the cellular distribution of cadmium in the gill, with some observations on localization of cadmium within the cells.

5.3.6 AN ELECTRON MICROSCOPE STUDY OF THE DISTRIBUTION OF HEAVY METALS IN THE GILL

The electron microscopy was performed in conjunction with Dr J.D. Popham, Department of Anatomy, University of Sydney, N.S.W., using oysters from the long term cadmium treatment system described in Chapter 2.

5.3.6.1 TISSUE CONTROLS

The tissue controls for the method of tissue preparation were:

ammonium sulphide, but with no developer (Plate 5.7(a) and (b)); and

no ammonium sulphide, but with developer (Plate 5.8(a) and (b)).

The lower power (Plate 5.7[a]) shows that a fine precipitate occurs along a line of mucus covering the microvilli, and in some other places, but cells are free from heavy precipitate.

Plate 5.7(b) is a higher power demonstrating that there is a fine precipitate over most of the tissue components of the section, and that the nuclear envelope has some slightly heavier precipitate

associated with it.

Plate 5.8(a) and (b) are sections of tissue in which the ammonium sulphide step has been omitted from the treatment. The mucus layer (presumably) has some heavy precipitate associated with it, including some near the cilia.

5.3.6.2 TESTS -BACKGROUND LEVELS OF CADMIUM

Sections of gill from oysters not exposed to cadmium are shown in Plates 5.9 and 5.10. The cytosomes do not display any grains in the control sections (Plates 5.7 and 5.8), so the appearance of grains over the cytosomes in the tests indicates the presence of high concentrations of heavy metals.

Oysters not exposed to cadmium treatment (control oysters from the long term cadmium treatment system) show a variable pattern of cellular metal accumulation. In some areas the gill is relatively free of metal (Plate 5.9[a]), while in other areas some cells display high levels of metal (Plate 5.9[b]). However, most heavy metals in these gill sections are either cytosome bound or in the cytoplasm of isolated cells. The isolated cells observed to contain metals in the cytoplasm are found in the centre of the gill filaments, in the region of the blood lacunae. Most metal-laden cytosomes are found in the peripheral area of the epithelial cells, close to their external surface.

In plate 5.9(a) the pattern of grains over the mucus is similar to that observed in the control (Plate 4.8) and should be treated as a false positive for the presence of metals. Also, although the grains in the nuclear envelope appear denser and larger than those in the control section (Plate 4.8), they may also represent a false

positive reaction.

The two cells containing very high levels of metal in Plate 5.9(b) are amoebocytes, described by Ruddell (1971a,b) as granular amoebocytes. The absence of metal in the nearby cells is striking, and demonstrates the localization of metals at the cellular level.

Plate 5.10(a) and (b) are high power electron micrographs of two cells. The first, 5.10(a), is a cell from Plate 5.9(b), and shows the highly localized sub-cellular accumulation of metals. Compared with the density of metal in the cytoplasm, very little metal can be seen in the nucleus of this cell. The second, 5.10(b), shows a cell with metal localized into the cytosomes (possibly lysozomes), with little in the rest of the cell. Nearby cells show very few grains.

The high level of metals observed in the gill cells from oysters not exposed to cadmium in the experimental system is consistent with the observation that oysters from the Clyde River contain approximately (by wet weight): 0.2 µg/g Cd, 15 µg/g Cu, 195 µg/g Zn, 0.9 µg/g Pb, 1.3 µg/g As (Mackay, et al., 1975).

5.3.6.3 TESTS - TREATED WITH CADMIUM

Oysters exposed to 50 µg/l cadmium in the flowing system for four weeks were used for comparison with those not previously treated with cadmium.

As with the oysters not cadmium treated, the pattern of metal accumulation was variable. Plate 5.11(a) shows a typical discontinous pattern of metal distribution. One cell is so heavily labelled that the contents are completely obscured, others are less heavily labelled with grains, and some are nearly unlabelled. The line of grains near

the bottom of Plate 5.11(a) corresponds with the basal lamina, and since grains are evident on the inside of this layer, it is unlikely that the dense layer of grains represents an artifact of fixative or ammonium sulphide diffusion into the tissue piece. Some cytosomes contain metals in apparently high concentrations, but other organelles do not display high levels of heavy metals.

Plate 5.11(b) shows a higher power view of the basal lamina, with a cytosome also containing a high level of metals.

Not all amoebocytes are observed to carry metals, and Plate 5.12(a) shows a granular amoebocyte (cell X) with very little metal, and another cell (cell Y) (possibly also an amoebocyte) with a heavy cytoplasmic load of metal. Plate 5.12(b) is a higher power of the latter cell, showing the cytoplasmic accumulation of metal, and the nearby cells with heavy cytosomal accumulation of metals.

Three types of amoebocytes are recognized by Ruddell (1971a, b) on the basis of morphological evidence. The discontinuous distribution of metals between the amoebocytes observed in the electron micrographs may reflect functional differences between the three types.

In the oysters treated with cadmium there were generally more metal-carrying cytosomes in the epithelial cells, mostly distributed in the apical portion of the cell, than in the untreated oysters.

Oysters exposed to cadmium for four weeks as already described were allowed to feed in running sea water for one week. The gills of these oysters were also examined using the sulphide precipitation technique.

Plate 5.13 shows that some cells from the gills of the

PLATE 5.7(a) and (b)

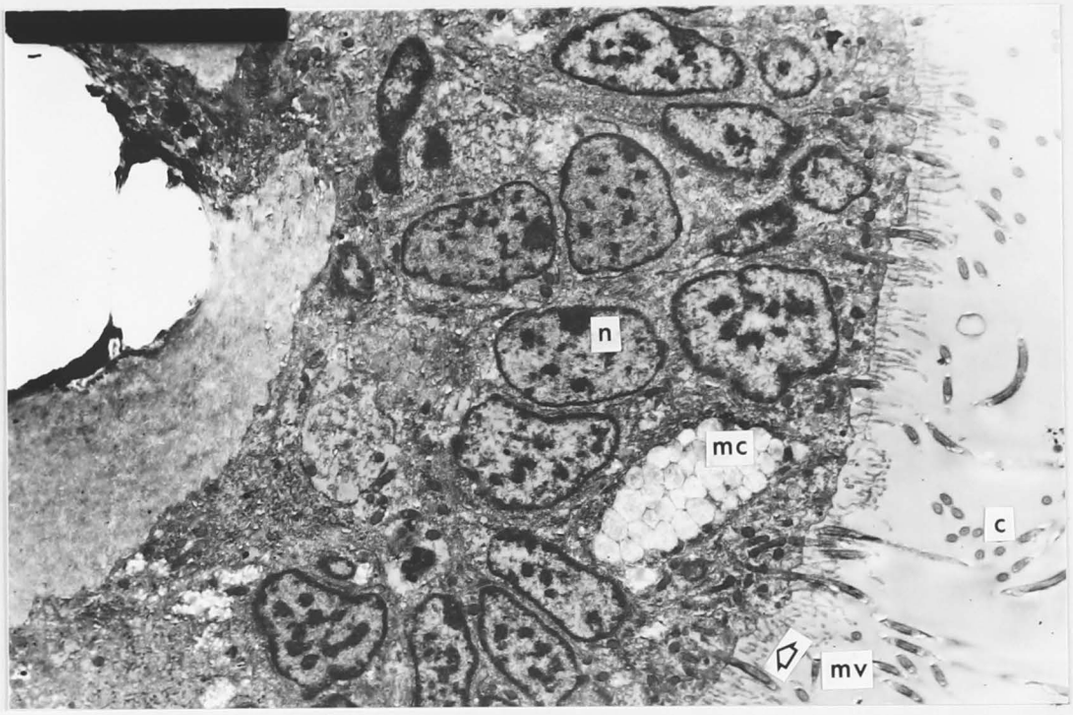
Electron micrographs of gill tissue. The sections have not been exposed to the quinone developer, but were exposed to the sulphide treatment, and serve as controls for the method of metal localization.

Magnification (a) 22,000 X

(b) 7,000 X

mc = mucous cell n = nucleus c = cilia

mv = microvilli



a

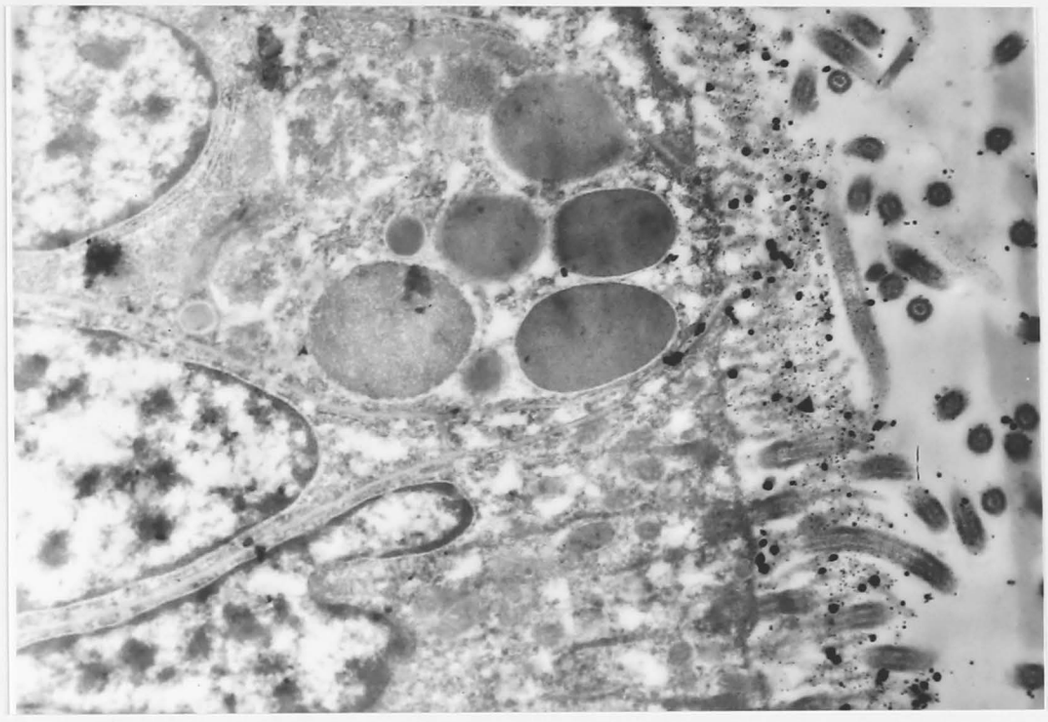
b

PLATE 5.8(a) and (b)

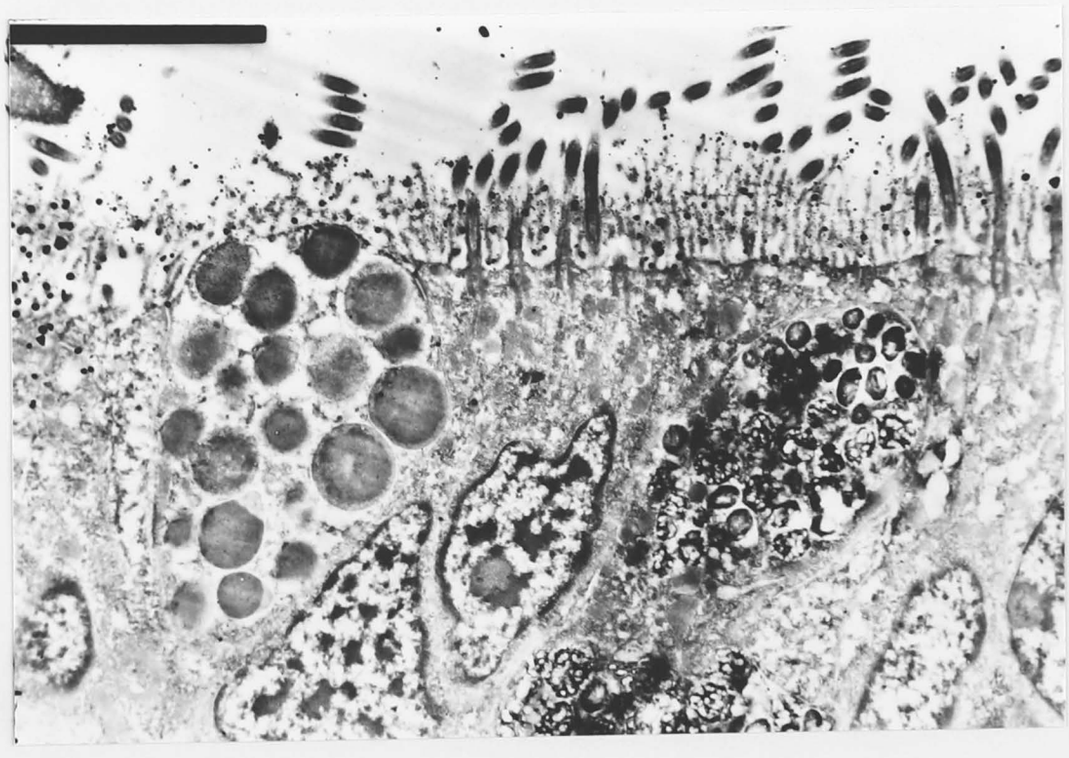
Electron micrographs of gill tissue. The sections have been exposed to the quinone developer, but not the sulphide treatment. These sections serve as controls for the method of metal localization.

Magnification (a) 28,500 X

(b) 11,400 X



a



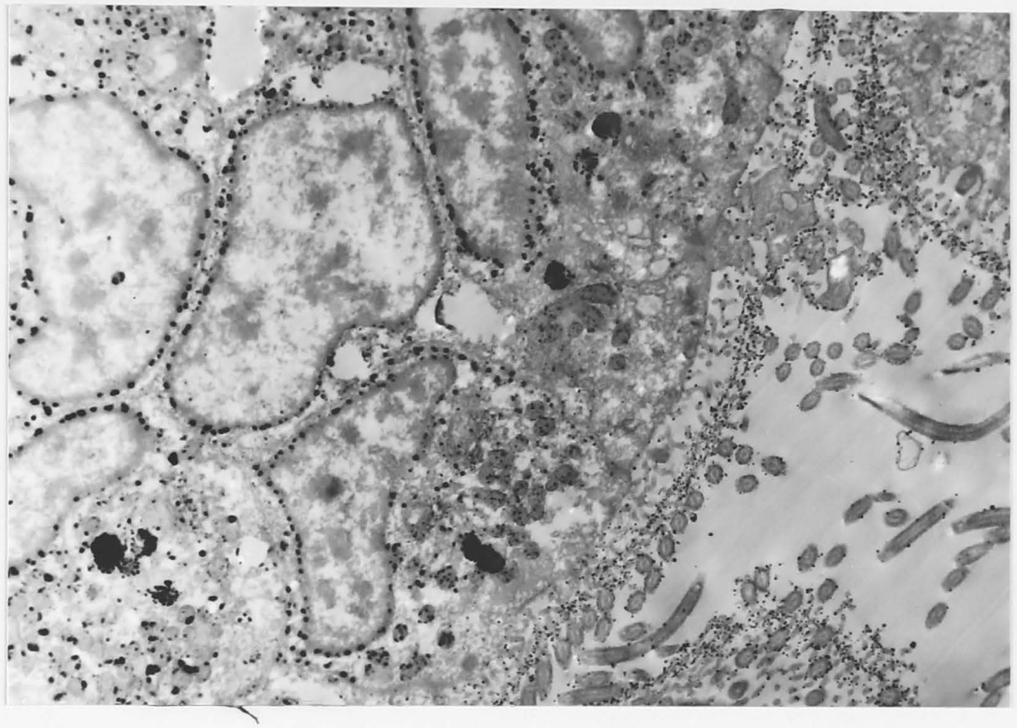
b

PLATE 5.9(a) and (b)

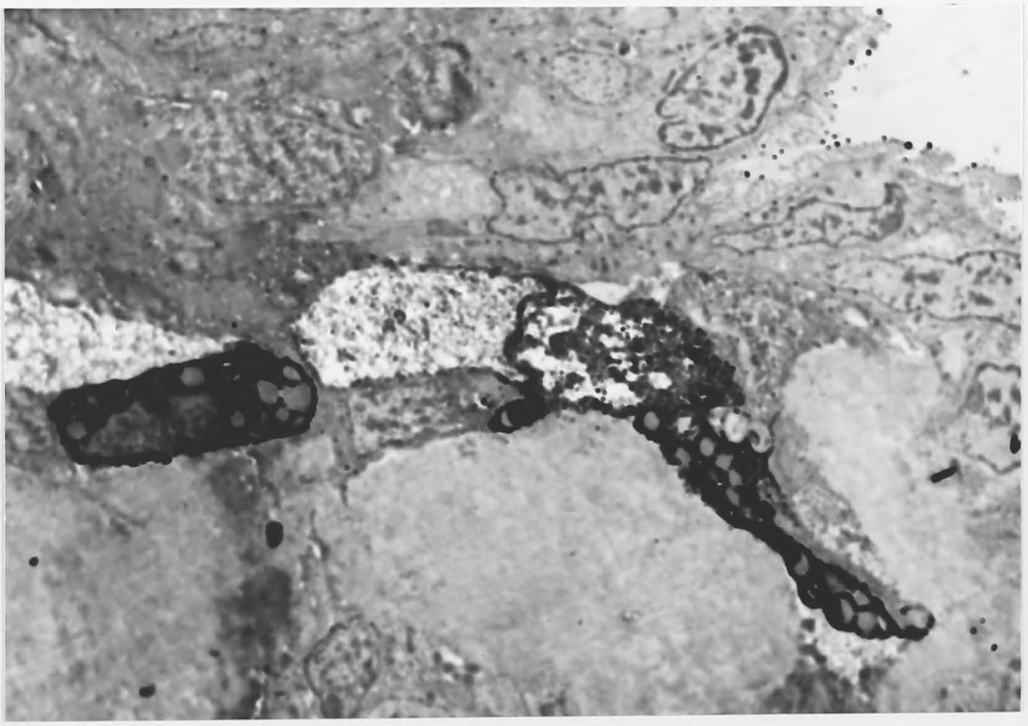
Electron micrographs of gill tissue. The oysters have not been exposed to cadmium. Heavy metals are localized using a sulphide precipitation technique. Electron dense deposits (grains) appearing on these sections represent areas of heavy metal localization if they do not correspond with grains in the control sections (Plates 5.7, 5.8).

Magnification (a) 18,000 X

(b) 8,000 X



a



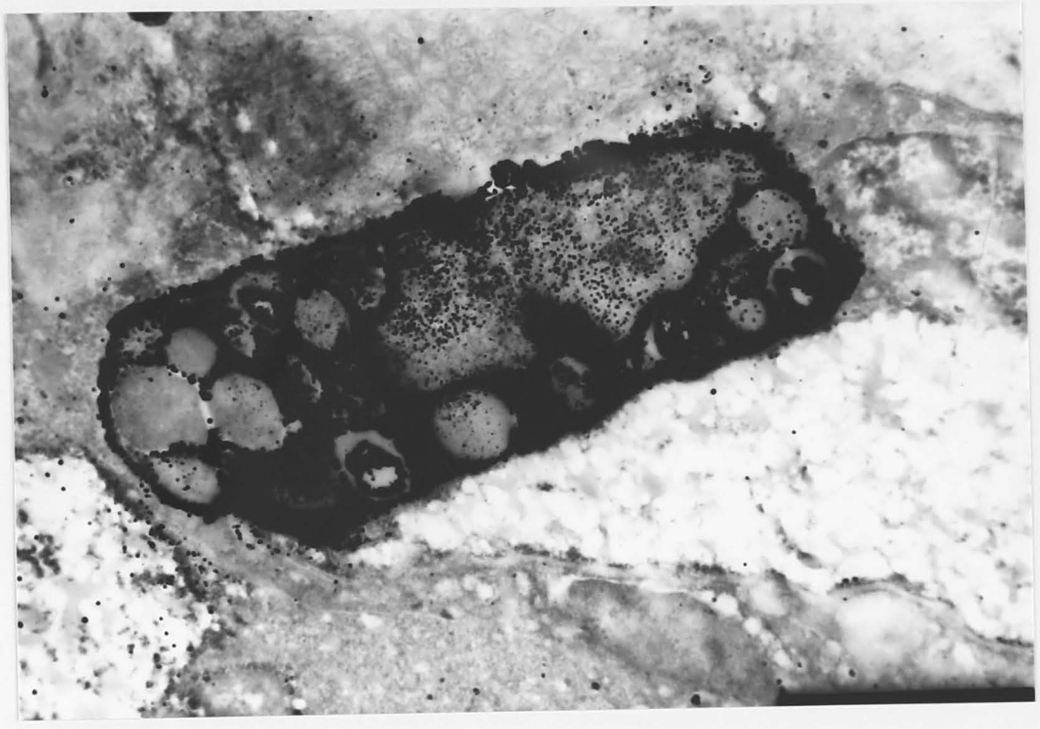
b

PLATE 5.10(a) and (b)

Electron micrographs of gill tissue. Two cells show metal localization. The oysters have not been exposed to cadmium.

Magnification (a) 22,000 X

(b) 22,000 X



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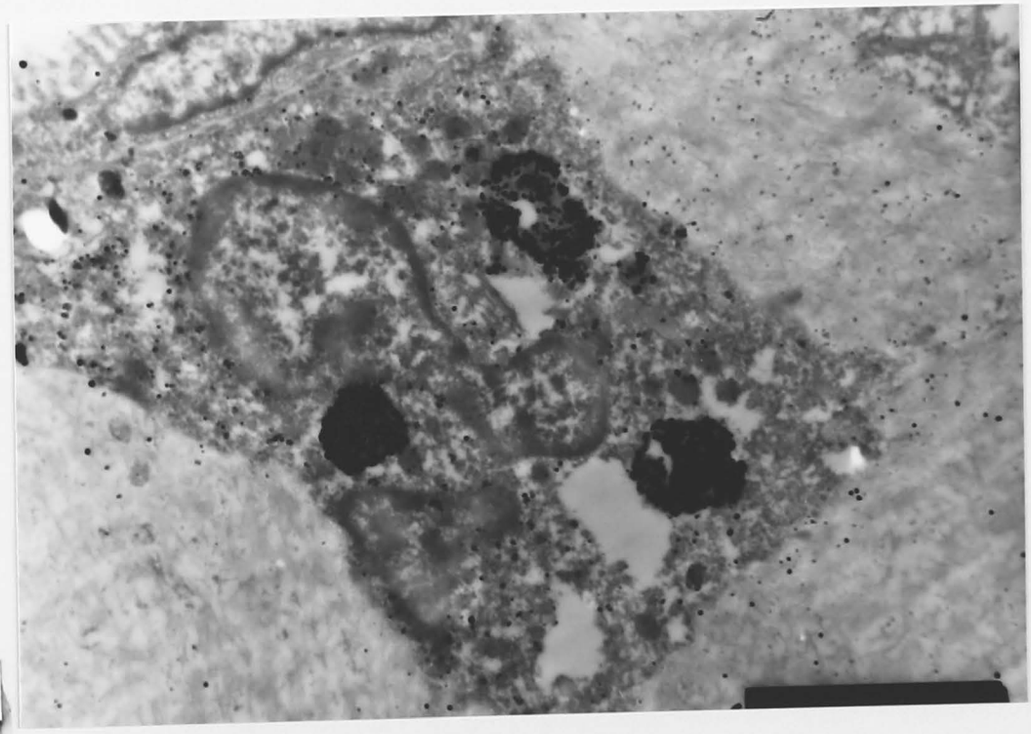


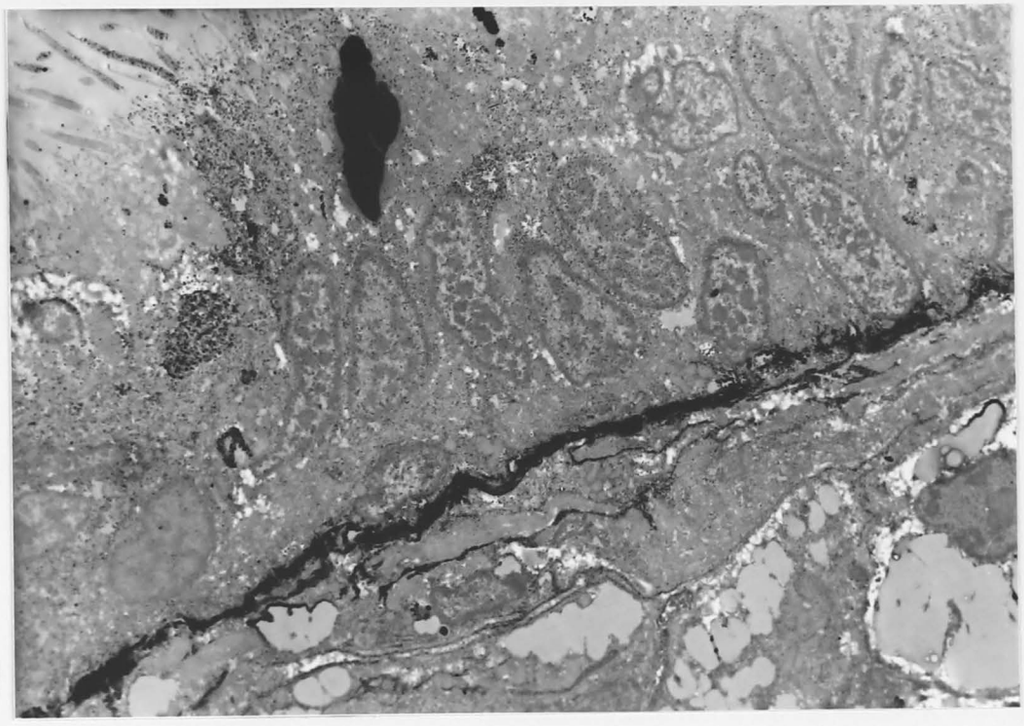
b

PLATE 5.11(a) and (b)

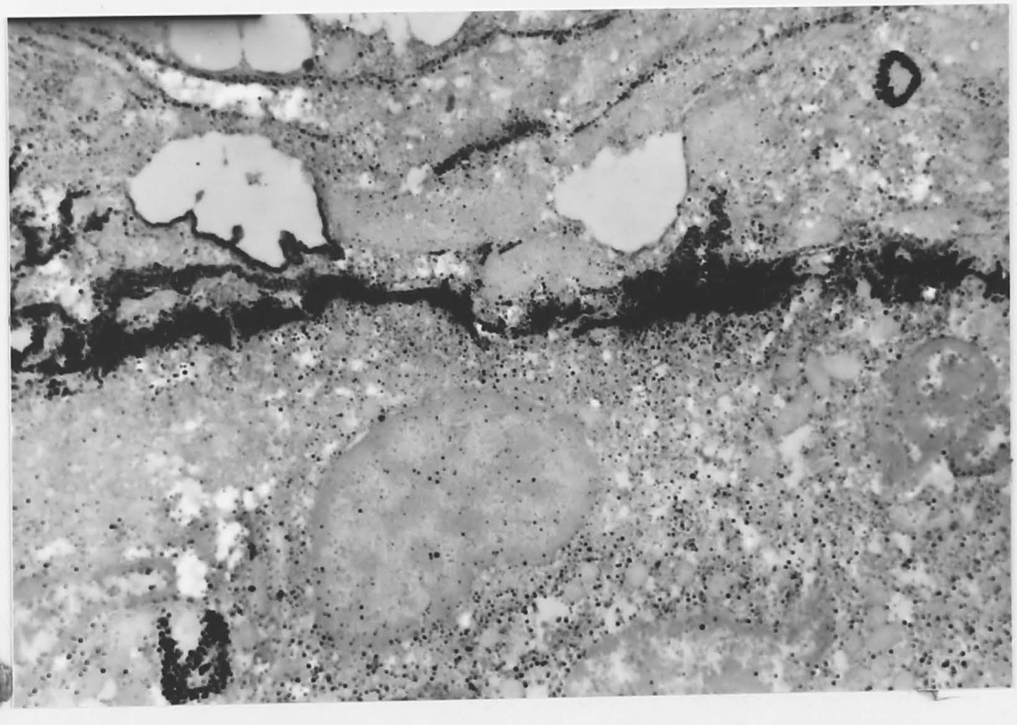
Electron micrographs of gill tissue. The oysters were exposed to 50 µg/l cadmium for four weeks in the flowing system. Heavy metals are localized using the sulphide precipitation technique.

Magnification (a) 10,000 X

(b) 18,000 X



a



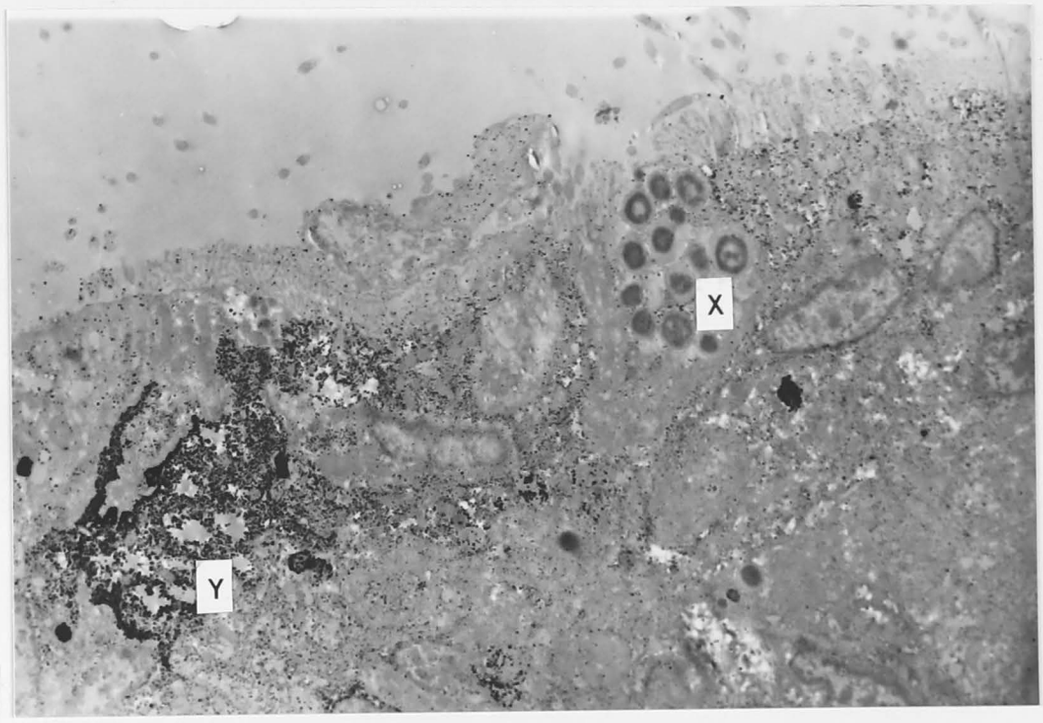
b

PLATE 5.12(a) and (b)

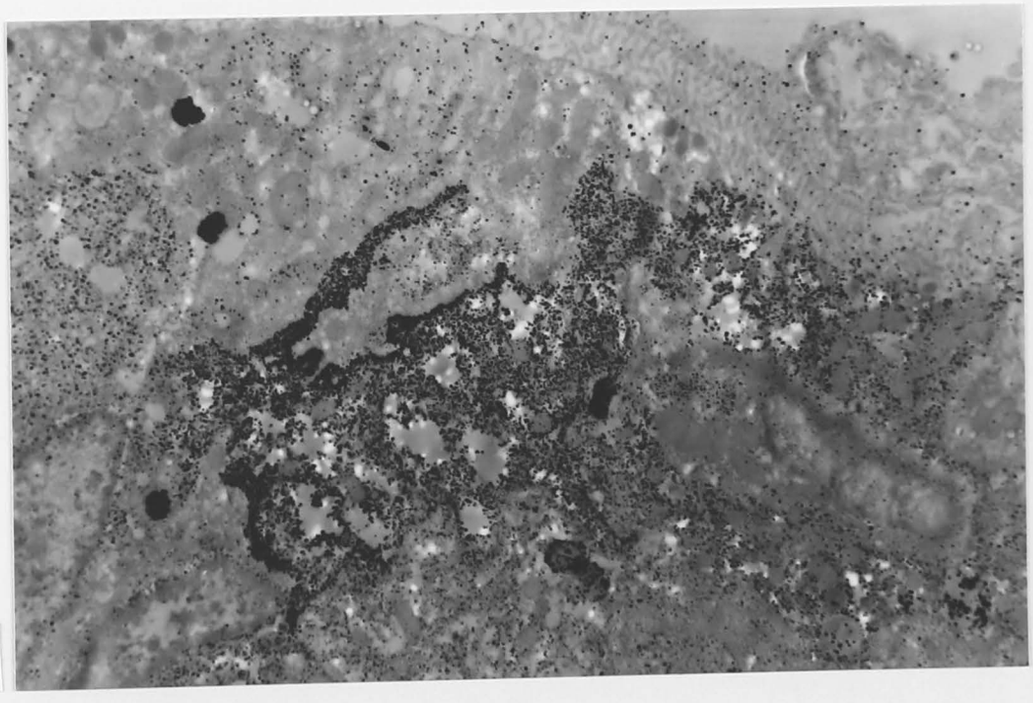
Electron micrographs of gill tissue. The oysters were exposed to 50 µg/l cadmium for four weeks in the flowing system. The cells marked X and Y are amoebocytes.

Magnification (a) 10,000 X

(b) 18,000 X



a



b

PLATE 5.13

Electron micrograph of oyster gill. The oysters were exposed to 50 µg/l cadmium for four weeks, then allowed to purge for one week in clean running sea water. Heavy metals are localized using the sulphide precipitation technique.

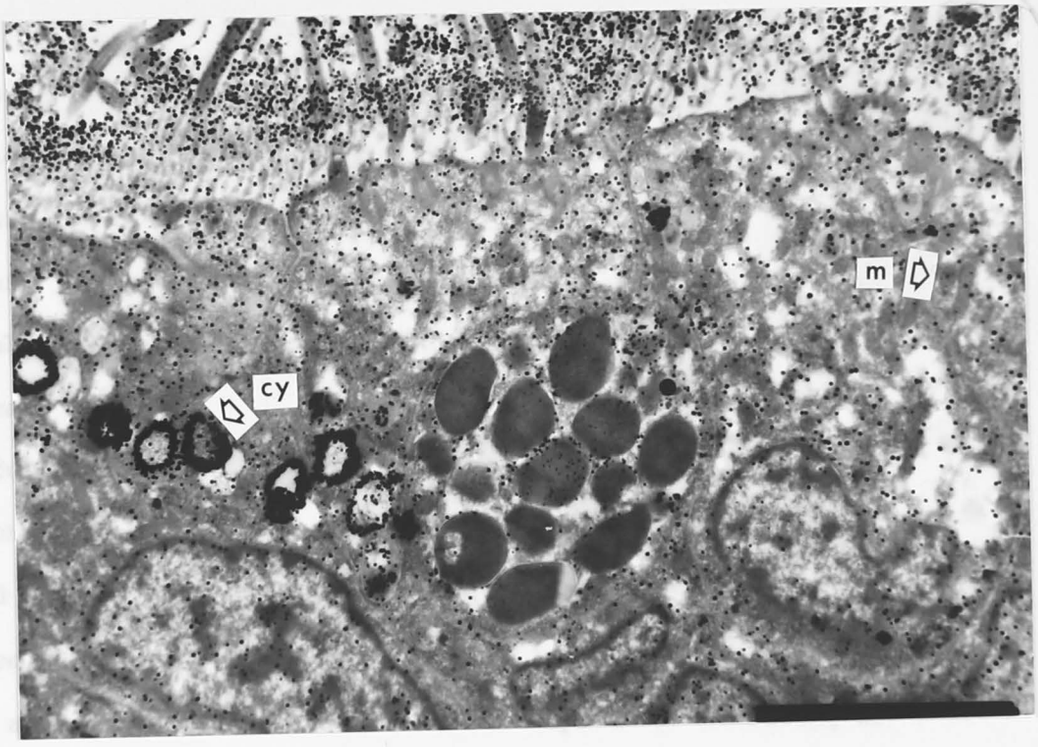
Magnification 18,000 X

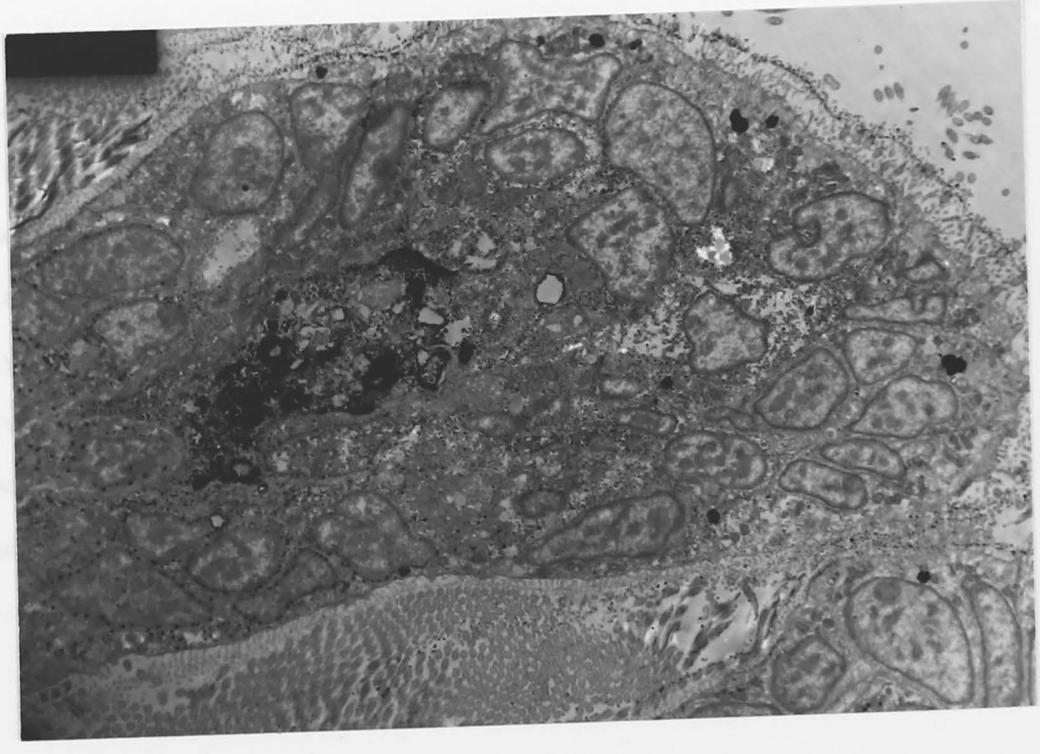
cy = cytosome m = mitochondria

PLATE 5.14

Electron micrograph of a gill filament from an oyster. The oyster has not been exposed to cadmium. Heavy metals are localized using the sulphide precipitation technique.

Magnification 8,000 X


cy
m



oysters allowed to purge for one week still contain a large quantity of metal which is associated with cytosomes. Although the mucus and cilia exhibit grains it is possible that they represent a false positive result (as previously discussed). It is possible that the mucus, either inherently or by sequestration, contains metal sulphides or other ligands capable of depositing silver grains. These possibilities deserve further investigation before any interpretation of the metal binding characteristics of either the cilia or the mucus could be attempted using the present technique. The mucous layer on the gill of *Mytilus edulis* contains iron (George, Pirie and Coombs, 1976) and it is commonly accepted that molluscan mucus is capable of adsorbing or absorbing metals (Pringle, et al., 1968).

From the results above, amoebocytes appear to be closely involved with cadmium binding at both natural and elevated environmental levels of cadmium. The other primary site of metal accumulation appears to be the cytosomes of the epithelial cells, also at both natural and elevated levels of cadmium.

It has long been known that amoebocytes can carry heavy metals (Galtsoff, 1953). Ruddell (1971b) and Ruddel and Rains (1975) describe the accumulation and transport of copper and zinc by oyster amoebocytes, and discuss the migration of amoebocytes through the mantle tissue, and their congregation near the tissue surface. Presumably, the amoebocytes are subsequently lost to the environment. The observation in this thesis that amoebocytes, probably in the blood system, contain high levels of metals is consistent with the observations of Ruddell (1971b).

During scanning of the electron micrographs from the

experiments reported here, numerous observations were made of metal-laden cells appearing in or near the centre of the gill filaments (for example see Plate 5.14). The area of the filament involved is probably the blood space, suggesting that the blood is used to transport the metal-laden amoebocytes to, from or through the gills. Numerous amoebocytes were observed apparently about to lyse into the external environment from the surface of the gill, although only in one case was an amoebocyte observed to carry significant amounts of metal. Also, Takatsuki (1934), Stauber (1950) and Tripp (1960) have examined the role of amoebocytes in digestion and defence, and suggest that while amoebocytes are commonly shed across the surface of the mantle, few are shed from the gill. Although the experiments reported here do not provide direct proof it appears that the amoebocytes are transported by the blood through the gills, but not shed across the epithelium. It follows that the metal loads of the amoebocytes will have little effect on gill function, since they are not in effective contact with most of the gill cells.

The cadmium treated oysters displayed an increase in the numbers of metal-laden cytosomes in the epithelial cells compared with the untreated oysters. It seems reasonable to assume that the cadmium has been absorbed from the water across the cell wall and localized in the cytosomes.

Fowler, Wolfe and Hettler (1975) report the involvement of cytosomes (in the mantle of *Mercenaria mercenaria*) with the binding of mercury and iron when the clam was challenged with mercury. The distribution of metal-laden cytosomes in the apical area of the epithelial cells of *Mercenaria* mantle is similar to that reported in

this section for cadmium in gill cells.

The treatment with cadmium did not cause any gross ultrastructural damage to the cells of the gill. A detailed examination was not undertaken because the sections were not suitably prepared for a careful study of the ultrastructure.

The observation that cadmium accumulates in the basal lamina of the gill is of unknown significance.

It seems probable that the cadmium found in the oyster gill after a challenge with cadmium is in two pools, that of the blood cells (amoebocytes) which is likely to be unavailable to the cells of the gill, and the more readily available intra-cellular pool. The cytosomes may act as scavengers for intra-cellular cadmium and remove much metal from the cytosol avoiding otherwise deletarious effects.

If the cytosomes of the epithelial gill cells resemble lysozomes in their function, it seems possible that the selective accumulation of metals by oyster cytosomes could be a protective mechanism not unlike that of mammalian lysozomes (Davies, 1973). In any case, the mechanism of metal scavenging by the cytosomes warrants a closer study.

5.4 GENERAL DISCUSSION

The autoradiographs demonstrate that cadmium is not evenly distributed throughout the body of the oyster. The gill, the gut and the heart/kidney show a greater retention of radioactive cadmium than the gonad or the adductor muscle. The observations are consistent with the results of Chapter 4, and with the recognized ability of these

organs to accumulate metals in other molluscs (Coughtry and Martin, 1976; Cunningham and Tripp, 1975a; Collins, Segar and Riley, 1971).

Since no gross differences are observed between the organ distribution patterns of cadmium after a soluble and a particulate challenge with cadmium, at least two mechanisms for the removal of cadmium from water may exist. During a challenge with soluble cadmium, the gut may receive cadmium in mucus while the gill may absorb cadmium directly from the water. Cadmium in circulating amoebocytes (accumulated from the gut and mantle) may also contribute to the apparent cadmium load of the gill. During challenge with a particulate source of cadmium the absorption of cadmium into amoebocytes in the gut and mantle may increase the level of cadmium circulating in the blood, and maintain the apparent cadmium load in the gill despite a reduced amount of cadmium absorbed by the gill cells directly from the water. These two mechanisms may maintain the apparent concentration of cadmium in the gill during both a soluble and a particulate challenge with cadmium.

Irrespective of the manner of accumulation (or the sites of uptake) a challenge with cadmium in two dissimilar forms has little effect on the total amount of cadmium absorbed. The two forms of cadmium used in these experiments, that is the soluble (probably ionic) species and the soluble form absorbed into insoluble bacteria can be accumulated with similar efficiency by *C. commercialis*. It is apparent that the gut is the most important single site of uptake of cadmium.

The epithelial cells of the gill appear to contain only a small fraction of the total cadmium load of the gill. The majority of

the cadmium is found in the gill amoebocytes. Since the two pools of cadmium (the cadmium bound in amoebocytes and the cadmium bound in the epithelial cells) may not exchange with each other, the heavy metals observed in the cytosomes of the epithelial cells may represent cadmium accumulated directly from the water. Although the latter pool of cadmium may be only small compared to the amoebocyte pool, it is likely only the cadmium absorbed directly from the water is capable of having toxic effects on the gill cells.

If the form of challenge with cadmium (that is soluble or particulate) affects the relative amounts of cadmium in the two pools of gill cadmium (but not the total) the toxicity may also be affected since I have postulated that the two pools may have different availabilities to the gill cells. If this is correct one ambient cadmium level may elicit different levels or types of toxicity, depending on the form of challenge. It is possible that a parallel may be drawn here with similar concepts in vertebrate toxicology, where different routes of challenge (subcutaneous, intravenous, oral, etc.) can result in different toxic effects.

The binding of most cadmium to the > 50,000 m.w. soluble fraction and the low speed membrane fraction (cell debris) by oysters pretreated for ten weeks with 50 µg/l cadmium suggests that the binding and detoxification of cadmium is not achieved by any single class of cellular components. The presence of a protein analogous to metallothionein seems unlikely. As the organ load increases, a change in the pattern of cadmium binding may represent the filling of cadmium binding sites of progressively lower affinities for the metal.

The binding of metals by the blood cells of oysters has long

been recognized (Galtsoff, 1964) and, from the results in this chapter, cadmium is no exception. The ability of amoebocytes to bind cadmium and to insulate effectively the other tissues against contact with cadmium may represent a mechanism for detoxification of cadmium. Ruddell and Rains (1975) show that amoebocytes may contain high concentrations of metals, and it is possible that, although specific metal binding proteins are not evident from the results in this chapter, an investigation of amoebocytes may reveal their presence.

The effect of cadmium on some functions of the gill is studied in Chapter 6, with a view to assessing effects that may be considered to be sub-lethal, rather than capable of rapidly causing death.

CHAPTER 6

THE EFFECT OF CADMIUM ON SOME GILL FUNCTIONS

6.1 INTRODUCTION

The gill of an oyster has two primary functions; first to create water currents through the mantle cavity, bringing in water from the external environment for feeding and respiration, and second to sort and trap food particles and transport them to the palps.

Gaseous exchange in the oyster gill is probably of secondary significance, since the circulation of blood through the gill is sluggish (Nelson, 1960). The mantle aids in gaseous exchange (Nelson, 1960; Galtsoff, 1964) and is supplied with accessory hearts to maintain adequate blood flow. The blood of *C. virginica* appears to lack any of the common molluscan respiratory pigments (Galtsoff, 1964), which suggests that the oxygen carrying capacity of the blood is sufficient in their absence. It seems unlikely that the highly complex oyster gill would have evolved without concurrent development of one of the otherwise common molluscan respiratory pigments if it is a mechanism whose function is to supply oxygen to the blood. It is thus probable that the complexity of gill structure is related more to feeding than to blood oxygenation.

Water from the external environment is passed through and over the gill, and is used for respiration, to remove excretory materials, to supply food, and as an aid in the dispersal of gametes during spawning. The oyster gill is therefore an efficient sorting, trapping and transport mechanism for food particles. Suitable particles

in the size range one to ten micrometres (μm) (Haven and Morales-Alamo, 1970) are trapped and transported to the palps by a combination of ciliary activity and mucous secretions. Unsuitable particles and particles outside the size range are rejected by the gill. Rejected material is usually entangled in a mucous net and transported by cilia of the gill and palps to a region of the mantle near the palps to await ejection. Ejection is accomplished by a rapid closing of the valves, often resulting in a sudden squirt of water. This "snapping" behaviour of *C. commercialis* can be readily seen by observing a bed of oysters covered by only a centimetre or two of water.

Sub-lethal or chronic interference with normal functioning of the oyster gill could have serious consequences on nutrition and spawning. Effects on nutrition are likely to have serious ecological consequences for both oyster populations and estuarine ecosystems. In New South Wales such effects would also result in significant losses in the commercial value of oyster crops. In addition, the hazards of normal environmental stresses to both cultured and natural populations could well be intensified by any impairment of gill function.

As described in previous chapters, the gill concentrates a significant fraction of the total body load of cadmium when the oyster is exposed to elevated environmental levels of cadmium. The gill therefore must be considered as a potentially "critical organ" for sub-lethal and chronic effects.

The activity of oyster gill cilia has been examined by several techniques in the past, including the "crawling" technique, the use of stroboscope, and by the "cone" method (Galtsoff, 1964). These techniques either require excision of the gill, or do not directly

assess ciliary activity. Removal of the right valve and excision of a piece of gill was considered, in the present study, too severe to allow detection of sub-lethal effects, so attempts were made to assess, by means of a stroboscope, the rate of beating of frontal and latero-frontal cilia on demibranchs *in situ*. The right valve was carefully removed to reveal the gill but all attempts failed to monitor the rate of cilia beating. Failure was due mainly to the flexing of the gill and difficulty in following particular groups of cilia.

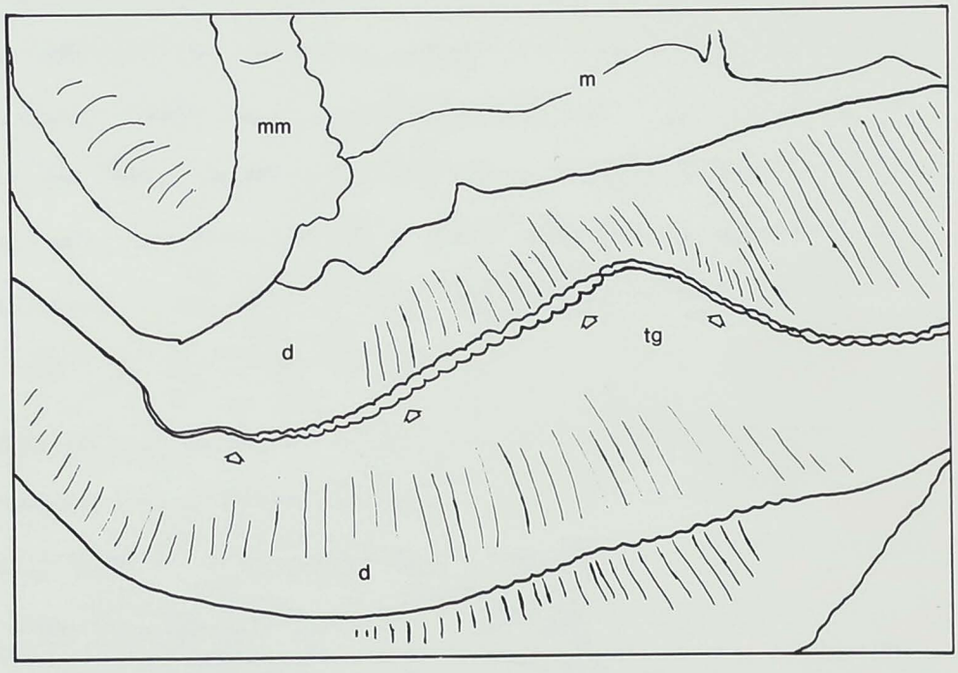
Subsequently two experiments were designed to assess gill function. First, the rate of particle transport along a defined ciliated area of the gill was assessed following a relatively long term exposure to cadmium. This was a quantitative attempt to study effects of cadmium on a particular function, and on specific gill cilia. The area chosen is known variously as the terminal groove (Galtsoff, 1964), marginal food collecting furrow (Nelson, 1960), and the marginal groove (Jorgenson, 1955). In any case, the groove referred to is located at the ventral (and anterior) margin of each demibranch, and functions in *C. commercialis* primarily in the transport of rejected material destined to be ejected by the oyster (pseudofaeces). I adopt the terminology of Galtsoff (1964) in this matter, and designate the groove the terminal groove (Plate 6.1).

The second experiment was designed to assess the rate of clearance of food size particles from suspension, a gill function of considerable significance in nutrition. The bacterium *Escherichia coli* falls within the probable size range for food particles (being approximately one micrometre in length), and preliminary trials indicated that oysters quickly cleared *E. coli* from suspension.

PLATE 6.1

Gill of an oyster.

m = mantle; mm = mantle margin; d = demibranch; tg = terminal groove (arrowed).


mm
m
d
tg
d

In filtered sea water containing a suspension of *E. coli* considerable quantities of faeces were observed. It therefore appeared that the oyster cleared the bacteria from suspension in a manner similar to that for food particles.

6.2 MATERIALS AND METHODS

6.2.1 RATE OF PARTICLE TRANSPORT

The hinge of the oyster to be observed was carefully broken using a small screwdriver, the right valve lifted, and the adductor muscle severed at its junction with the shell. The mantle remained undamaged, except near the adductor muscle.

Observations were made using a binocular microscope at 15.75X magnification. The gill was exposed by placing the oyster in an evaporating dish with 500 ml of filtered sea water, and folding back the right mantle. Under these conditions the gill cilia continued to beat for several days, given daily changes of water. Providing that measurements were made soon (15 min) after a change of water the rate of particle transport after three days was similar to that observed within two hours of opening the oyster.

The rate of transport of carmine particles was assessed by carefully placing two or three drops of a carmine/sea water suspension near the gill surface. The carmine particles were quickly gathered in by the gill and transported to either the terminal groove or the food groove at the base of the gill. The time, in seconds, required for a particle in a continuous mucous string to be carried along the terminal groove was recorded. The two reference points for the time measurements were the edges of the field of view along a diameter. A

minimum of ten times were recorded at each observation, and frequently several observations were made in quick succession. Free particles of carmine were transported considerably faster than particles in a continuous mucous string, so measurements were not made unless a continuous mucous string was evident. Care was taken to avoid overstimulation of the gill with an excess of carmine particles.

All observations were made at 20°C (in a constant temperature room) and the pH of the sea water used was adjusted to 6.5 before commencement of the experiment. The oysters were exposed for 13 weeks to the relevant cadmium concentrations in the standard flowing sea water system. Oysters were normally used direct from the aquaria in which they were stored. In the "anaerobic" experiment however, the oysters were removed from water 24 to 32 hours prior to observations. Two series of observations were conducted on "aerobic" animals, one within four hours of opening and another between 14 and 20 hours after opening. "Anaerobic" animals were examined at one hour after opening only.

6.2.2 CLEARANCE OF *E. COLI* FROM SUSPENSION

E. coli labelled with ^{14}C were prepared by adding one loop of wild type *E. coli* (provided by Dr. W.L. Nicholas) and approximately three microcuries of ^{14}C isoleucine (L[U-^{14}C] isoleucine, 330 mCi/mmol, Amersham) to 50 ml of minimal *E. coli* culture medium (Davis, Dulbecco, Eisen, Ginsberg and Wood, 1967, p. 139). The mixture was incubated at 37°C for 48 hours with occasional shaking, and then centrifuged (2300 G) for 15 min. The pellet was resuspended in 0.2% formalin for one hour at 4°C. The bacteria were then washed twice with 0.45 µm filtered sea water. The final pellet was resuspended in 50 ml of

filtered sea water, and an aliquot of this suspension was added to each experimental tank. Ten ml of the suspension contained between 110,000 and 280,000 cpm.

After 24 hours in filtered sea water the oysters responded to the addition of *E. coli* by actively feeding for several hours. No activity measurements were attempted, but animals were periodically observed (without disturbance) throughout the experiments to ensure continued feeding.

Water samples (5 ml) were taken at specified intervals and centrifuged at 2300 G for 15 min to partition the sample into supernatant and pellet fractions. The supernatant fraction contained some label, probably ^{14}C released from lysed bacteria and some ^{14}C from exchange reactions, whilst the pellet fraction contained the bacteria from the total water sample. No $^{14}CO_2$ production could be detected from either the labelled bacterial suspension or from an oyster filtering the bacteria from this suspension, over a period of 24 hours.

Two ml of either the supernatant or the pellet fraction (resuspended in 2 ml) were added to 1 ml distilled water and 10 ml of PCS Scintillant (Amersham/Searle). The mixture was counted by scintillation in a Packard Tricarb counter, and the results corrected for background. The addition of distilled water is necessary to maintain the sample in an homogenous condition and allow efficient counting.

The experimental controls employed oyster shells (oysters shucked and the valves reassembled) to assess the loss of label from the water column by other than oyster mediated methods. In the short term experiment readings were taken throughout the experiment, but in

the long term experiments only initial and final readings were taken in the controls.

Experiments were conducted at 19°C in shallow one litre polyethylene containers, using an experimental volume of 500 ml, and with no aeration. In all experiments the sea water used was adjusted to the relevant cadmium concentration with stock solutions of $CdCl_2.2\frac{1}{2}H_2O$ in distilled water (pH 3). Volumes of stock cadmium solutions added were always less than 1 ml.

At the conclusion of the experiments oysters were shucked and weighed, and the visceral mass dissected away from the rest of the animal. These remaining soft parts, comprising the muscle, mantle, gill and palps, were weighed and solubilised in NCS Tissue Solubilizer (Amersham/Searle) at 50°C for one week. Faeces were collected from the bottom of the tanks by taking up faecal strings with a pasteur pipette and transferring them to a cotton wool plug to be rinsed with distilled water. The cotton wool and faeces were then soaked in PCS scintillant for two weeks, and finally counted. This technique for examination of the faeces was effective only in picking up large faecal strings, and proved less than satisfactory. The results should be considered as semi-quantitative.

6.2.2.1 SHORT TERM CADMIUM EXPOSED OYSTERS

Oysters were purchased locally, scrubbed free of encrusting organisms and acclimated for one week to laboratory conditions. Prior to exposure to labelled *E. coli* three animals were held for 24 hours in 500 ml of filtered sea water at each of 0, 10, 50 and 100 µg/l cadmium. At time 0 hours fresh cadmium/sea water solutions were added to the experimental tanks and the labelled *E. coli* suspension added and

thoroughly mixed. The initial water sample was taken before feeding commenced. No faeces samples were collected in this experiment.

6.2.2.2 LONG TERM CADMIUM TREATED OYSTERS

Oysters were exposed to cadmium in the standard running sea water system for six and a half weeks prior to use.

Two experiments were conducted. Two experimental tanks (duplicates) were used for each cadmium treatment, although one tank contained two oysters and the other contained three. The two tanks within a cadmium treatment were considered as replicates in the analysis of results. The results were pooled for both experiments, but since the same five oysters were used for the two experiments the sample size remains at five oysters for each cadmium treatment.

If the apparatus for the long term exposure of oysters to cadmium had been located near Canberra it would have been feasible to follow the effects of cadmium on *E. coli* clearance on a weekly basis. The two experiments reported in this section were performed within five days of each other, and no attempt was made to follow, or correct for, the time course of cadmium effects.

6.3 RESULTS

6.3.1 PARTICLE TRANSPORT

Means of observations on the rate of carmine transport in the terminal groove are recorded in Table 6.1. Each observation was recorded from a different oyster and was taken 15 min after a change of water in all cases.

In the initial four hours of observation all but one of the 50 µg/l cadmium treated oysters displayed no transport that could be

measured. However, after a further 10 to 15 hours, activity recovered in one other oyster, and two rates were recorded (Table 6.1). Some cilia activity was observed in various parts of the gill of the 50 µg/l treated group, but it was irregular and appeared to be asynchronous. During the time of observation large areas of cilia were recorded as not beating at all in the 50 µg/l treated animals. By contrast, in the control group of animals, once ciliary activity resumed after a disturbance ciliary activity could be observed in all parts of the gills. No attempts were made to quantify these observations.

The 50 µg/l treated oysters kept for 24 to 32 hours out of water ("anaerobic") displayed rates of carmine transport similar to those in the 0 and 10 µg/l groups, when measured one hour after opening (Table 6.2).

6.3.2 CLEARANCE OF *E. COLI* FROM SUSPENSION

The presence of ^{14}C in soft parts other than the visceral mass, the appearance of label in the faeces (Table 6.3), and the maintainence of ^{14}C levels in the supernatant fraction indicate that isoleucine from ingested *E. coli* is incorporated into oyster tissue.

6.3.2.1 SHORT TERM

Although results shown in Figure 6.1 and Table 6.4 indicate that 24 hours exposure to cadmium appears to progressively depress the maximum rate of removal of *E. coli* from suspension, a χ^2 test on the data (transformed to cumulative % clearance) reveals a significant difference only between the controls and the 100 µg/l cadmium treated group.

However, this experiment shows that approximately 10% of the label added as *E. coli* suspension is left in a soluble form in the

TABLE 6.1

The rate of particle transport by "aerobic" oysters. One observation per oyster, means ± s.e.m. Oysters were exposed to cadmium for 13 weeks.

WITHIN 4 HOURS OF OPENING

[Cd] (µg/l)	0*	10*	50
time (s)	n = 4	n = 4	n = 6
	27.9 ± 3.3	33.0 ± 4.3	25 (one recording)

14 TO 24 HOURS AFTER OPENING

[Cd] (µg/l)	0*	10*	50
	n = 3	n = 3	n = 5
time (s)	26.9 ± 2.4	36.3 ± 6.1	24.9 (two recordings)

* No significant difference by t test.

TABLE 6.2

The rate of particle transport by "anaerobic" oysters. One observation per oyster, means ± s.e.m. Oysters were exposed to cadmium for 13 weeks.

ONE HOUR AFTER OPENING

[Cd] (µg/l)	0*	10*	50*
	n = 3	n = 5	n = 4
	27.6 ± 4.2	30.5 ± 2.6	26.45 ± 4.7

* No significant difference by t test.

TABLE 6.3

The incorporation of ^{14}C from *E. coli* into oyster tissue and faeces. Oysters were exposed to cadmium for 24 hr (short term) and 6½ weeks (long term).

A. SHORT TERM:

TISSUE: means of 2 oysters/group

[Cd] (µg/l)	total cpm	cpm/g wet wt.
0	4,975	2,596
10	5,913	3,145
50	3,239	1,748
100	4,390	2,783

B. LONG TERM:

TISSUE: means of 4 oysters/group

[Cd] (µg/l)	total cpm	cpm/g wet wt. ± s.e.m.
0	2,534	9,520 ± 3,321
10	1,259	5,061 ± 1,055
50	1,044	4,214 ± 691
100	334	1,343 ± 236*

FAECES: means ± s.e.m. of 4 experimental tanks

[Cd] (µg/l)	cpm
0	5,177 ± 556
10	3,733 ± 723
50	5,536 ± 1,783
100	10,023 ± 1,365

* Significantly different from controls and [50] group.

TABLE 6.4

Clearance of *E. coli* from suspension by short term (24 hr) cadmium exposed oysters. Single observations.

PELLET FRACTION: cpm/g wet wt.

[Cd] (µg/l)	0 hr	1 hr	2 hr	4 hr	6 hr	10 hr	14 hr	20 hr	24 hr
0	33.2	10.9	10.0	8.1	6.1	5.0	4.7	4.5	3.9
10	43.0	20.0	13.6	9.5	7.3	5.7	5.0	4.7	4.2
50	30.3	16.1	12.0	9.0	7.4	5.5	4.2	3.7	8.3
100	43.3	39.2	25.0	11.9	8.2	6.6	5.9	5.3	4.9
Control (cpm only)	579.9	529.0	476.9	600.8	534.8	504.7	496.6	427.2	-

CUMULATIVE PERCENT CLEARANCE*

[Cd] (µg/l)	0 hr	1 hr	2 hr	4 hr	6 hr	10 hr	14 hr	20 hr	24 hr
0	0	67.2	69.9	75.6	81.6	84.9	85.8	86.4	88.3
10	0	53.5	68.4	77.9	83.0	86.7	88.4	89.1	90.2
50	0	46.9	60.4	67.3	75.6	81.9	86.1	87.8	89.9
100	0	9.5	42.7	72.5	81.1	84.8	86.4	87.8	88.7

* No significant differences between the control and the 10 and 50 µg/l groups. The 100 µg/l group is significantly different from the control ($P < 0.05$, χ^2 test).

MAXIMUM RATES OF REMOVAL: Expressed as % of cpm/g at 0 time.

0 (control) = 67.2% 10 µg/l = 53.5% 50 µg/l = 46.8% 100 µg/l = 32.7% (between 1 & 2 hr)

water, and that this level does not change over a 24 hour experimental period (Table 6.5). Control tanks containing only oyster shells and bacteria showed a slight drop in insoluble level in the water column over 20 hours. The slight drop may be caused by settling of bacteria in the control tanks, which would not occur to the same extent in the experimental tanks because of oyster feeding currents.

6.3.2.2 LONG TERM

The effects of long term cadmium treatment on clearance of *E. coli* by oysters are shown in Table 6.6 and Figure 6.2 The results were further analysed by transformation of the data from cpm/g wet wt. (Table 6.6) to a cumulative percentage clearance of label, with the 0 time being regarded as 0% loss of label (Table 6.7). The different values observed at time 0 in Table 6.6 are caused primarily by the different wet weights of oysters in each group, since identical volumes of *E. coli* suspension were dispensed to each experimental tank. Table 6.7 shows the % of counts remaining in the water column (= bacteria in suspension) at specific sampling times.

Analysis of variance on the transformed data reveals significant effects of both time and cadmium treatments on the removal of *E. coli* from suspension. Comparison amongst means using a method of least significant difference (LSD, Sokal and Rohlf, 1969, pp. 227 and 235) shows that all cadmium treated groups removed *E. coli* from suspension at significantly slower rates than the control group in the first two hours of feeding. Within this time there are no significant differences between the rates of the cadmium treated groups. After three and four hours feeding there is no significant difference between the 10 µg/l and the control group, although the 50 µg/l group has a

TABLE 6.5

Clearance of ^{14}C from soluble fraction by short term (24 hr) cadmium exposed oysters. Single observations.

SUPERNATANT FRACTION: cpm/g wet wt.

[Cd] (µg/l)	0 hr	1 hr	2 hr	4 hr	6 hr	10 hr	14 hr	20 hr	24 hr
0	3.3	2.9	3.0	2.9	3.3	3.3	3.7	3.7	3.5
10	4.1	4.1	3.7	4.0	4.3	4.2	4.2	4.5	4.2
50	3.0	2.7	2.7	2.7	2.8	3.0	3.0	3.1	3.4
100	4.7	4.5	4.4	4.0	4.2	4.4	4.9	4.6	4.6
Control (cpm only)	62.6	72.7	76.4	67.5	74.3	72.0	73.6	74.5	63.3

TABLE 6.6

Clearance of *E. coli* from suspension by long term (6½ wk) cadmium exposed oysters.

A. POOLED RESULTS FROM TWO EXPERIMENTS, FIVE OYSTERS PER CADMIUM TREATMENT, FOUR OBSERVATIONS PER CADMIUM TREATMENT. MEAN ± s.e.m.: cpm/g wet wt.

TIME HR	Control	10 µg/l Cd	50 µg/l Cd	100 µg/l Cd
0	125.9 ± 25.0	109.2 ± 18.5	152.7 ± 40.3	173.4 ± 35.3
0.5	89.1 ± 16.2	91.8 ± 16.1	133.3 ± 40.5	155.1 ± 33.9
1	67.7 ± 12.7	78.5 ± 18.5	117.5 ± 40.0	140.7 ± 45.2
1.5	57.5 ± 10.8	68.9 ± 15.5	103.9 ± 36.2	124.7 ± 27.6
2	48.4 ± 8.1	53.4 ± 7.7	91.8 ± 31.6	112.1 ± 23.1
3	41.3 ± 7.8	49.7 ± 12.0	77.7 ± 26.4	94.8 ± 17.8
4	34.1 ± 5.1	41.2 ± 7.4	67.9 ± 19.3	83.9 ± 15.7
8	27.2 ± 5.2	27.9 ± 3.5	44.9 ± 11.6	54.6 ± 8.7
10	26.6 ± 5.1	25.2 ± 2.5	40.4 ± 11.1	49.5 ± 7.6

B. MAXIMUM RATES OF REMOVAL: expressed as units/hr as a % of cpm/g at 0 time.

Control = 49.5 10 µg/l = 30.25 50 µg/l = 27.75 100 µg/l = 22.75

TABLE 6.7

Clearance of *E. coli* from suspension by long term (6½ wk) cadmium exposed oysters. Results from Table 6.6 transformed to cumulative percent clearance.

TIME HR	Control	10 µg/l	50 µg/l	100 µg/l
0	0	0	0	0
0.5	29.6(a)	16.0	16.5	12.0
1	49.5(a)	30.25	27.75	22.75
1.5	54.25(a)	38.5	36.0	28.0
2	61.0(a)	46.0	43.25	34.5
3	66.75	56.0	51.5(b)	44.5(c)
4	71.5	72.25	55.25(b)	49.25(c)
8	76.5	73.0	70.0	65.25
10	77.25	75.25	73.0	69.0

(a) = Significantly different from the cadmium treated groups at this time

(b) - Significantly different from the control

(c) = Significantly different from 10 ppb and control groups

ANOVA - SOURCE OF VARIATION

Factor	d.f.	Ms	F	P
time (A)	8		132.3	< .001
Cd (B)	3		28.48	< .001
A x B	24		1.03	ns
error	108	71.36		

L.S.D. = 11.95

FIGURE 6.1

The clearance of *E. coli* from suspension by cadmium treated oysters (24 hr). Each point represents a single observation. The *E. coli* were labelled with ^{14}C, and the results are expressed as the cpm of the pellet fraction (obtained by centrifugation of a 5 ml water sample)/g wet weight of oyster/tank. The results are from Table 6.4.

FIGURE 6.1

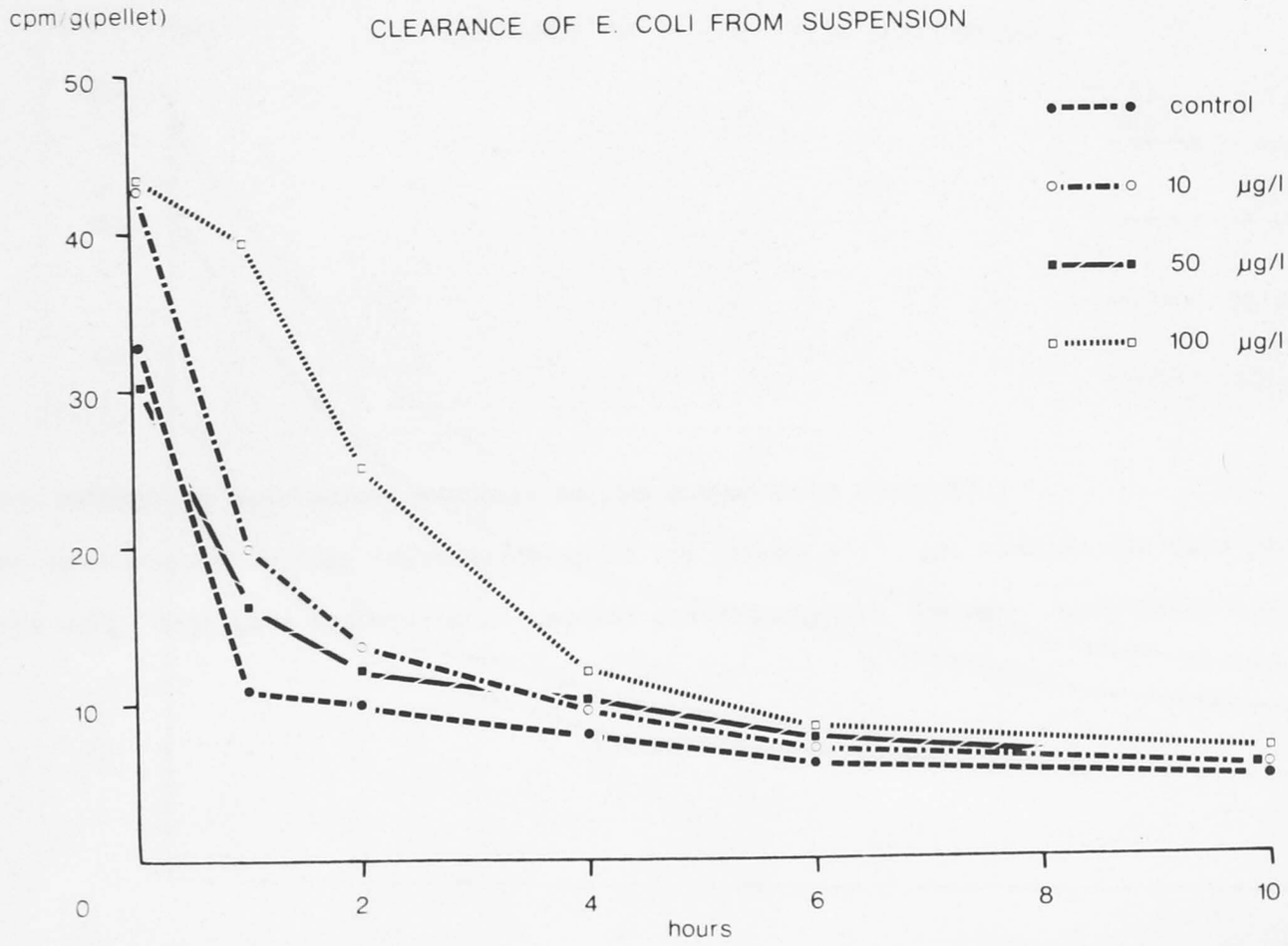
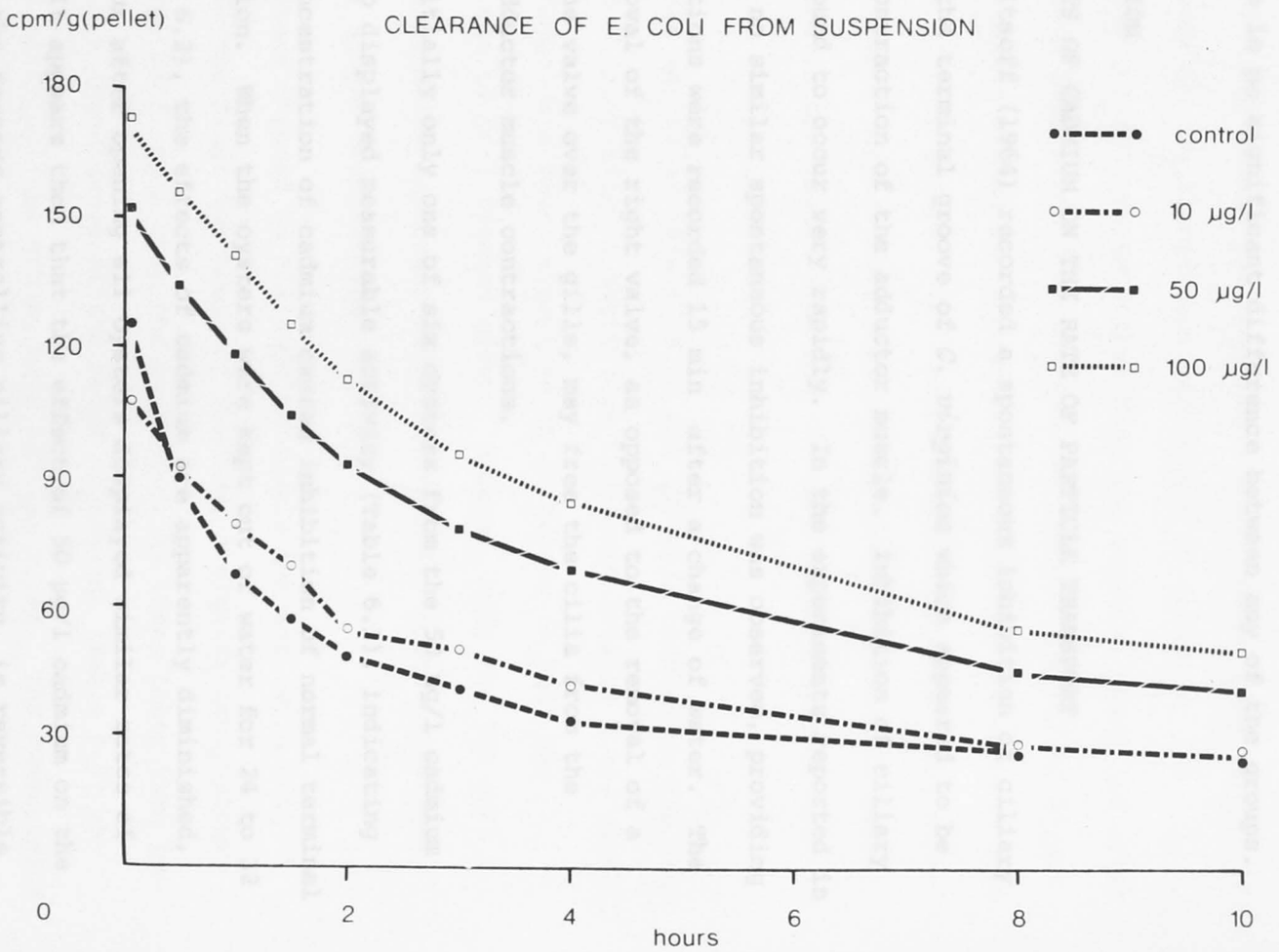

cpm/g(pellet)
CLEARANCE OF E. COLI FROM SUSPENSION
50
40
30
20
10
0
2
4
6
8
10
hours
control
10 µg/l
50 µg/l
100 µg/l

FIGURE 6.2

The clearance of *E. coli* from suspension by cadmium treated oysters (6½ wk). Each point represents the mean of four observations. Other information is as for Figure 6.1. The results are from Table 6.6, and Table 6.7 contains a statistical analysis of the transformed results.

FIGURE 6.2

CLEARANCE OF E. COLI FROM SUSPENSION



significantly slower rate than the controls. Also after three and four hours feeding the 100 µg/l group has a significantly slower rate than the 10 µg/l group, but not the 50 µg/l group. After eight hours feeding there is no significant difference between any of the groups.

6.4 DISCUSSION

6.4.1 EFFECTS OF CADMIUM ON THE RATE OF PARTICLE TRANSPORT

Galtsoff (1964) recorded a spontaneous inhibition of ciliary activity in the terminal groove of *C. virginica* which appeared to be related to contraction of the adductor muscle. Inhibition of ciliary action was found to occur very rapidly. In the experiments reported in this section no similar spontaneous inhibition was observed, providing that observations were recorded 15 min after a change of water. The complete removal of the right valve, as opposed to the removal of a section of the valve over the gills, may free the cilia from the effects of adductor muscle contractions.

Initially only one of six oysters from the 50 µg/l cadmium treated group displayed measurable activity (Table 6.1), indicating that this concentration of cadmium caused inhibition of normal terminal groove function. When the oysters were kept out of water for 24 to 32 hours (Table 6.2), the effects of cadmium are apparently diminished, since one hour after opening all oysters displayed similar rates of transport. It appears then that the effect of 50 µg/l cadmium on the cilia, or on the factors controlling ciliary activity, is reversible given a sufficiently strong stimulus.

6.4.2 CLEARANCE OF *E. COLI*

Isoleucine is an essential amino acid for oysters, and must be

supplied in the diet or by microbial gut flora (McGilvery, 1970, p. 376). Only a small amount of ^{14}C isoleucine is incorporated into tissues from the digestion of *E. coli* (Table 6.5), consistent with the likely physiological demand for this amino acid. After long term exposure to cadmium the significant depression of uptake of ^{14}C isoleucine into the soft parts (other than the visceral mass) indicates either a reduced absorption from the gut, or reduced ability to digest the bacteria, since ultimately almost all the bacteria are removed from suspension by all the groups. The faeces samples, although only semi-quantitative, could support this possibility since considerably more label was found in the faeces from the 100 µg/l treated oysters than in the controls.

There may be an effect of 50 and 100 µg/l cadmium on the rate of removal of *E. coli* after a short term treatment (Figure 6.1), since the initial rates of removal appear to be different to both the controls and the 10 µg/l treated group. However, only the 100 µg/l group is significantly different from the controls, probably because they are only single observations.

Oysters exposed to all cadmium levels for six and a half weeks show, in the first two hours of feeding, a significantly slower rate of removal of *E. coli* than the controls. At times later than two hours there is a convergence of the rates of removal (Table 6.7) so that after 10 hours all the groups have similar rates. The convergence of rates is caused by the closed design of the experiment, without an infinite supply of bacteria to maintain a constant *E. coli* concentration. A better simulation of natural conditions would involve keeping the level of labelled bacteria nearly constant within the

experimental tanks.

The experiments described in this section can be related to the field situation by comparison of the maximum rates of removal of *E. coli* in the first one or two hours of feeding. A measure obtained in this manner is likely to be a good assessment of comparative feeding performance in the field. When the maximum rates of removal are compared (Table 6.6) it is clear that the long term cadmium treated oysters all suffer reduced feeding efficiency.

The rate of particle clearance from suspension may be affected by both changes in the rate of water transport and changes in the filtration of particles by the gill. Although water transport is controlled by the gill, the mantle and the adductor muscle (Galtsoff, 1964), the evidence from 6.3.1 suggests that the experiments reported in this chapter assess the effects of cadmium on the gill cilia rather than the other organs mentioned. However, there is no evidence to distinguish between the effects of cadmium on water transport by the gill, and effects on particle sorting by the gill. Both could be affected. However, regardless of the mechanism, the result of a relatively long term treatment with cadmium, at levels as low as 10 µg/l, is a decrease in the efficiency of feeding by the oyster.

Cultured populations of *C. commercialis*, and probably many natural populations, spend only a small time feeding, because of their intertidal habitat. An effect on feeding such as that resulting from the long term treatment with cadmium could well prove to be a considerable disadvantage for oysters. The impact on commercial cultivation would be readily observed, since the growth rate is an important parameter and is usually followed closely by the oyster

farmer. However, the effects in natural populations could well pass unnoticed in the absence of an environmental stress of unusual magnitude.

Normally, oysters progressively build up glycogen reserves to allow for energy generation during times of environmental stress (Galtsoff, 1964). An interference with this process is likely to be one result of a reduction in feeding efficiency, which would ultimately reduce resistance to environmental stress. Similarly, gonad maturation relies heavily on metabolism of glycogen reserves, so reproductive success could be indirectly reduced by cadmium treatment. In the light of these observations an examination of the energy status of oysters treated with cadmium may prove to be of some significance. Chapter 7 describes some preliminary experiments to examine the effect of cadmium on the energy metabolism of the gill.

CHAPTER 7

THE EFFECT OF CADMIUM ON INTERMEDIARY METABOLISM IN THE OYSTER GILL

7.1.1 INTERMEDIARY METABOLISM IN THE OYSTER

Intermediary metabolism is the utilization of substances in a defined sequence of degradative steps to produce energy for various cellular functions. The degradative sequence forms high energy phosphate bonds (or their equivalents), recycling many compounds, and consistently maintaining the metabolic integrity of the cell.

Glycolysis, which degrades glycogen or glucose to lactate, is a major pathway of intermediary metabolism during anaerobiosis. Under aerobic conditions, lactate is not formed. Pyruvate, as its precursor, is utilized in the citric acid cycle, which, when linked to a suitable electron acceptor, serves to couple the degradation of glucose to the production of CO_2 and the utilization of oxygen, releasing considerable quantities of energy. These pathways of energy production form the basis for energy production in most other animals.

In the oyster, intermediary metabolism has been studied mainly in an attempt to elucidate the anaerobic functioning of the component pathways. Aerobic metabolism has been largely left to extrapolation, inference or speculation from the evidence provided by studies in other animals. Given the known variation of some enzyme parameters between organs of *Crassostrea* species, and the difficulties of extrapolation from adductor muscle to mantle, extrapolation from other animals is, at best, dubious. It is often necessary to make

these extrapolations, but pitfalls for the unwary are great and data relating to other organisms must be interpreted with due caution.

The oyster gill is not a favorite organ for biochemical study (when compared to adductor muscle) and for this reason, for reasons discussed in Chapter 1 ("critical organ" concept), for its role as a metal accumulator and for ease of sampling, the gill was chosen for investigation in this study.

The oyster gill has a more complex function than the gill of many other invertebrates. Not only does it function in gaseous exchange, but it also discriminates between food particles to be ingested or rejected (Galtsoff, 1964). The sorting mechanisms displayed by the oyster gill are the basis for nutrition of the animal, and aid in defence against bacterial invasion. It is conceivable that chronic effects of cadmium in the gill could contribute to the gradual demise of the animal in any number of ways. For instance, interference with mucous production may affect response to turbidity or bacterial challenge, and reduction of sorting efficiency may influence the amount of glycogen stored by the animal and hence resistance to any subsequent environmental stress.

7.1.2 AEROBIC PATHWAYS*

The literature supplies a confusing and, in the case of oyster gill, an incomplete picture of the aerobic pathways of intermediary metabolism. A summary of the evidence from the literature is depicted in the accompanying diagram (Figure 7.1). Contradictory

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* Abbreviations used throughout this chapter are contained in Table 7.7.

or doubtful evidence will be discussed. It should be emphasized here that Figure 7.1 represents my interpretation of the facts available and, in my opinion, the most probable sequence of steps.

The pathway for glucose degradation is similar to the classical scheme for glycolysis in vertebrate muscle. The oxidative decarboxylation of pyruvate to acetyl co-enzyme A and the subsequent generation of NADH in the citric acid cycle is an aerobic mitochondrial process in vertebrates and provides for the production of high energy phosphate bonds in the Electron Transport System using oxygen as the terminal electron acceptor. Under aerobic conditions, in the classical scheme, lactate is not normally formed, being recognized as a typical anaerobic end product. In this case anaerobic lactate production serves to regenerate NAD for the continued degradation of glucose to pyruvate, allowing substrate level phosphorylation to continue.

Pyruvate dehydrogenase has not been reported from oysters, although the pathway from glucose to pyruvate is not in doubt. Humphrey (1950) and Usuki and Okamura (1956) have reported various intermediates and enzymes of glycolysis from species of *Crassostrea*. Simpson and Awapara (1965) demonstrated the direct conversion of pyruvate to glucose in a reversal of the glycolytic pathway in *Rangia cuneata* mantle, and inferred from this the feasibility of the normal forward direction of glucose degradation. Engel and Neat (1970) have reported hexokinase, glucose -6- phosphate dehydrogenase, phosphofructokinase, fructose -6- diphosphatase, pyruvate kinase and lactic dehydrogenase activities from *Mercenaria mercenaria* (intertidal bivalve) gill, and also inferred the potential degradation of glucose

FIGURE 7.1

A summary of the pathways of intermediary metabolism in the oyster gill.

Net movement of carbon in the direction indicated by the arrows results in the following reactions at the sites shown, thus

1: oxidation of NADH to NAD

2: reduction of NAD to NADH

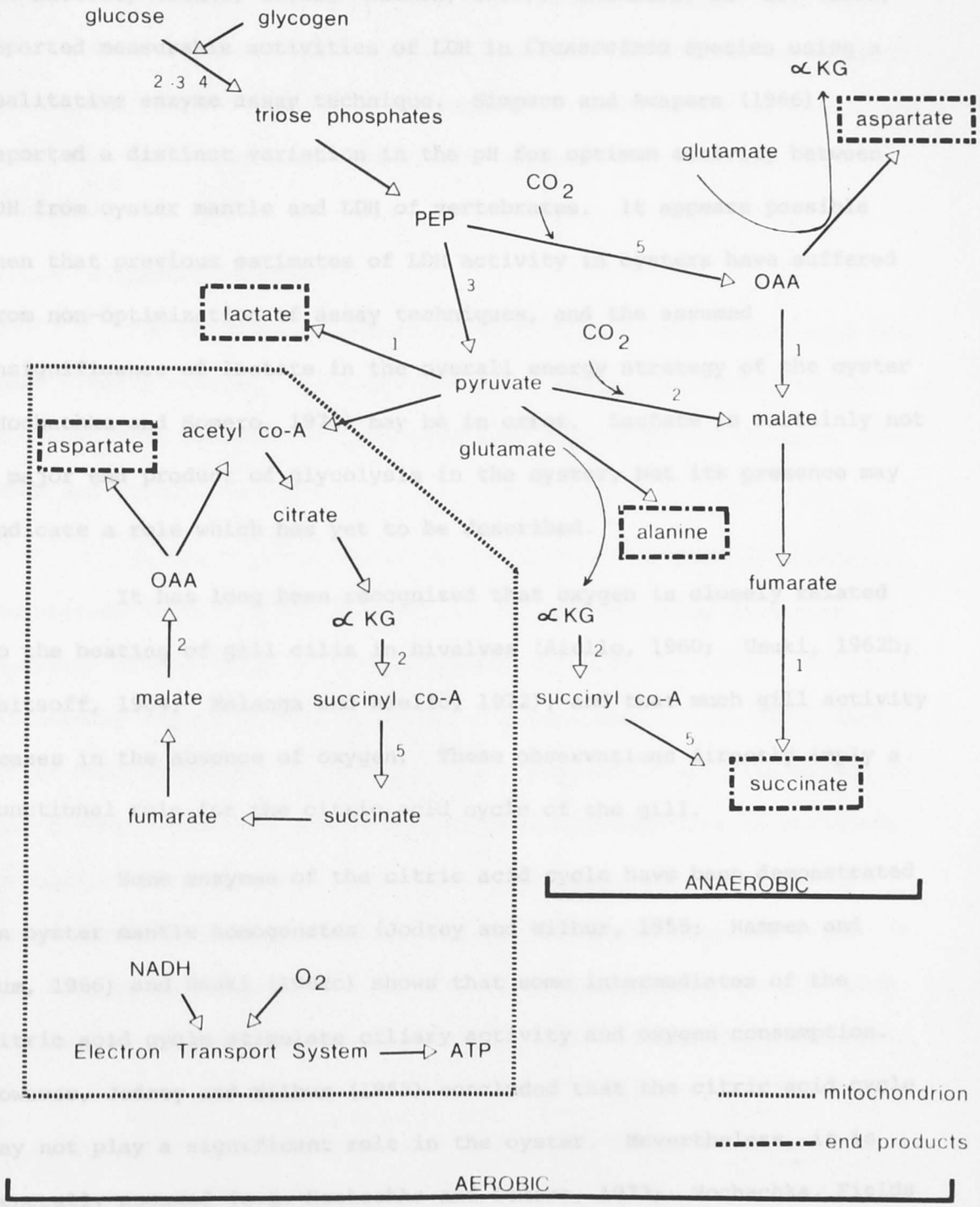
3: phosphorylation of ADP to ATP

4: conversion of ATP to ADP

5: phosphorylation of GDP to GTP

FIGURE 7.1

INTERMEDIARY METABOLISM IN THE OYSTER GILL



by the classical glycolytic pathway. Lactic dehydrogenase has been reported from oyster gill, although generally the enzyme displays very low activity (Usuki, 1962a; Hammen, 1969). Chambers, et al. (1975) reported measurable activities of LDH in *Crassostrea* species using a qualitative enzyme assay technique. Simpson and Awapara (1966) reported a distinct variation in the pH for optimum activity between LDH from oyster mantle and LDH of vertebrates. It appears possible then that previous estimates of LDH activity in oysters have suffered from non-optimization of assay techniques, and the assumed insignificance of lactate in the overall energy strategy of the oyster (Hochachka and Somero, 1973) may be in error. Lactate is certainly not a major end product of glycolysis in the oyster, but its presence may indicate a role which has yet to be described.

It has long been recognized that oxygen is closely related to the beating of gill cilia in bivalves (Aiello, 1960; Usuki, 1962b; Galtsoff, 1964; Malanga and Aiello, 1972), and that much gill activity ceases in the absence of oxygen. These observations directly imply a functional role for the citric acid cycle of the gill.

Some enzymes of the citric acid cycle have been demonstrated in oyster mantle homogenates (Jodrey and Wilbur, 1955; Hammen and Lum, 1966) and Usuki (1962c) shows that some intermediates of the citric acid cycle stimulate ciliary activity and oxygen consumption. However, Jodrey and Wilbur (1955) concluded that the citric acid cycle may not play a significant role in the oyster. Nevertheless, it is generally assumed (e.g. Hochachka and Somero, 1973; Hochachka, Fields and Mustafa, 1973) that, in the aerobic situation, energy is derived from the classical linkage of glycolysis to the citric acid cycle, and

the production of ATP through an electron transport system using oxygen as the terminal electron acceptor. However, the literature fails to demonstrate unequivocally the "normal" functioning of the citric acid cycle at a rate commensurate with the rate of consumption of oxygen. In the absence of convincing evidence to the contrary, the existence of a functional citric acid cycle in the gill must be accepted.

7.1.3 ANAEROBIC PATHWAYS

The pathways of anaerobic metabolism in the oyster have been more clearly defined, and are known to be similar, in many respects, to the anaerobic pathways in some parasites (Hochachka and Mustafa, 1972; Behm and Bryant, 1975).

It has been suggested that during the aerobic to anaerobic transition in the oyster an "all or nothing" switching mechanism operates at the level of PEP (Hochachka and Mustafa, 1972). It would, in effect, switch the main pathway of degradation at the PEP branch point from pyruvate, to OAA formation. Instead of pyruvate kinase in the former situation, phosphoenolpyruvate carboxykinase is utilised. Cytosolic PEPCK is widely distributed throughout bivalves, and the properties of PK and PEPCK which contribute to the proposed switch in the flow of carbon have been studied in oyster adductor muscle (Chen and Awapara, 1969; Simpson and Awapara, 1966; Hochachka and Mustafa, 1973; Mustafa and Hochachka, 1973a, 1973b). However, PEPCK activity in oyster gill is low (Mustafa and Hochachka, 1973a) which introduces the possibility that PEP to OAA may not be the primary anaerobic pathway in the oyster gill.

Pyruvate carboxylase activity has been reported from oysters

by Jodrey and Wilbur (1955) although Simpson and Awapara (1964) were unable to detect it in *Crassostrea*. DeZwaan and Marrewijk (1973) report that mitochondrial PC activity is widely distributed in *Mytilus edulis*, but if the enzyme does exist in the oyster its quantitative role has yet to be described.

Malate can be converted to pyruvate in the oyster gill by "Malic enzyme", which is thought to function primarily in the direction of pyruvate formation *in vivo*, despite its ready reversibility (Hochachka and Mustafa, 1973). Therefore, although the route is not clearly defined, pyruvate is formed anaerobically (Simpson and Awapara, 1966; Chen and Awapara, 1969).

It has been suggested that a primary fate of pyruvate produced anaerobically is transamination with glutamate, producing alanine and αketo-glutarate. αKG may then be utilized to produce succinyl Co-A, and ultimately succinate, generating NADH and GTP (Hochachka and Mustafa, 1972). The presence of GPT and citric acid cycle activity, and the energy advantages accrueing to the cell, suggest that this pathway is utilized in the oyster gill (Hochachka, 1975).

The probable production of succinate from αKG and pyruvate is supplemented by the more traditional anaerobic pathway to succinate, that is: PEP → OAA → mal → fum → succ.

Figure 7.1 shows that the major end products of anaerobic metabolism are succinate and alanine. The carbon derives from glucose (or glycogen) and the amino group is probably supplied by glutamate. Glutamate is readily available in molluscan tissues, since amino acids

are in plentiful supply (Baginski and Pierce, 1975), and amino-transferase activities maintain the concentrations of individual amino acids (Hammen, 1968). In *Crassostrea* the level of glutamate is approximately 5 nmol/g tissue (Awapara, 1962). Amino acids have additional importance in the metabolism of oysters in that they maintain cell and organism integrity during times of osmotic stress (Hammen, 1968; Baginski and Pierce, 1975). Their levels are actively regulated (Pierce, 1970) and change with fluctuations in salinity. The aerobic and anaerobic production of amino acids is therefore likely to be a vital step in intermediary metabolism.

7.1.4 DISTRIBUTION OF PATHWAYS

In spite of the "all or nothing" switch theory of Hochachka and Somero (1973) for oyster adductor muscle, it seems highly likely that the oyster gill displays simultaneous aerobic and anaerobic pathway activity during periods of plentiful oxygen supply.

Although oxygen is closely coupled to cilia activity in the gill (Usuki, 1962c), some types of cilia are able to beat anaerobically, and are cyanide insensitive (Gray, 1924, cited in Malanga and Aiello, 1972; Aiello, 1960; Usuki, 1962c). The oyster gill utilizes considerable amounts of endogenous glycogen (Usuki, 1962c). If the cells of the gill which are active during anaerobiosis ("anaerobic" cells) are organized and adapted for function primarily in anaerobiosis, and if they lack significant "aerobic" pathways, it is possible that they could continue to function "anaerobically" during aerobic conditions. "Anaerobic" end products would then build up within these cells, before being transported to other cells for further metabolism, which would account for observations of "anaerobic" end

products accumulating during aerobic conditions. Cells having primarily an "aerobic" function would have only a residual capacity for anaerobic metabolism, in order to maintain cellular integrity during anaerobiosis.

This proposal for intercellular compartments for "aerobic" and "anaerobic" pathways, and their simultaneous function during aerobic conditions in the oyster gill, is supported in principle by Livingstone and Bayne (1974). These authors consider that, in the mantle of *Mytilus edulis*, there is likely to be simultaneous function of both types of pathways, in contrast to the "all or nothing" concepts of Hochachka and Somero (1973), and that the relative flux through the pathways is controlled by the degree of tissue hypoxia.

An alternative hypothesis is that the pathways may be isolated intracellularly, perhaps mediated by the distribution of mitochondria. The little evidence available does not allow discrimination between the two possibilities.

7.2 EFFECT OF CADMIUM ON INTERMEDIARY METABOLISM

7.2.1 GENERAL

Vallee and Ulmer (1972) summarize many of the known stimulatory and inhibitory effects of cadmium on enzymes from a wide variety of species, and discuss the possibilities of effects on membranes and mitochondria. A number of these enzymes are associated with intermediary metabolism, and the effects of cadmium on some of them are exerted *in vivo* as well as *in vitro*.

This raises problems concerning transport of the highly active cadmium ion into the cell, and its subsequent transport

throughout the organism. The avidity with which cadmium binds to proteins with available SH groups is thought to be of great biological significance (Vallee and Ulmer, 1972). It may well prove to be a basis for the competitive antagonism of cadmium in zinc metabolism, as well as for the transport and detoxification of cadmium by vertebrates.

In vertebrates, cadmium is absorbed and transported within the body, and has toxic effects on various organs (Friberg, et al., 1974). Effects of cadmium on intermediary metabolism have been most commonly examined in rabbits, rats and mice.

7.2.2 THE ACUTE EFFECTS OF CADMIUM

Acute or "subacute" levels of cadmium elevate GOT and GPT activities in rabbits (cited in Friberg, et al., 1974). Sporn, et. al. (1970) demonstrated that high levels of cadmium *in vivo* interfered with rat liver oxidative phosphorylation, and that the effect could also be demonstrated *in vitro* at lower levels of cadmium, although not *in vivo*. However, at the lower levels of cadmium intake, aldolase activity decreased, phosphorylase b activity decreased, and phosphorylase a activity increased. Vallee and Ulmer (1972) concluded that the available evidence supported an effect of cadmium on carbohydrate metabolism prior to electron transfer.

In conditions of acute cadmium intoxication (60mg/Kg) Merali, et al. (1974) reported that rat liver PEPCK, PC, FDPase, and G6Pase activites were elevated by cadmium, as were blood glucose and serum urea concentrations, but that liver glycogen was depressed. Diamond and Kench (1974) demonstrated a significant cadmium interference with respiration prior to electron transfer. A study of GOT by Gould and Karolus (1974) in the cunner (*Tautogolabrus*

adspersus) at acute levels of cadmium showed a 59% depression of GOT activity. Chronic effects were not considered. Jackim, Hamlin and Sonis (1970) reported a depression of liver acid phosphatase activity in killifish (*Fundulus heteroclitus*) by a 27 mg/l acute *in vivo* dose of cadmium. These reports indicate that there may be little consistency of effect of cadmium in different animals and that, depending on experimental conditions, intermediary metabolism may be either stimulated or inhibited.

7.2.3 THE CHRONIC EFFECTS OF CADMIUM

At chronic levels of cadmium intoxication Merali, et al. (1974) concluded that the same effects observed in the acute study (quoted above) were present, although lesser in magnitude. Similarly, Yoshikawara (1974) demonstrated that rabbits chronically intoxicated with cadmium had elevated GOT, GPT, and LDH activities. Bilinski and Jonas (1973) found in trout (*Salmo gairdneri*) that, at acute levels, the ability of the gill to oxidize lactate to CO_2 was strongly impaired, although at lower levels no effect was observed, despite the death of test animals. Hiltibran (1971) found, in the bluegill, that low levels of cadmium (*in vitro*), inhibited liver mitochondrial succinic oxidase and αKG oxidase. Christensen (1975) found, at low *in vivo* levels of cadmium, an increase in trout GOT activity.

It seems clear, then, that a number of enzymes involved in carbohydrate and amino acid metabolism can be effected by cadmium, although the direction and magnitude of the changes are not predictable, and may depend on experimental factors as yet undescribed. In any case, the levels of cadmium used, their route of administration and the parameters subsequently examined vary widely from study to

study, which makes the interpretation and correlation of such results very difficult.

7.2.4 OYSTERS

The literature suggests that oysters possess both an aerobic and an anaerobic intermediary metabolism. Since many of the steps involved require divalent metal cations, it would appear likely that they could be affected by cadmium accumulation. Table 7.1 lists some enzymes requiring zinc. Some enzymes use thiol groups in their structure or function, and, without requiring a specific metal ion for activity, may be inhibited by heavy metals in one of several ways (Dixon and Webb, 1964, p. 341). Unfortunately, the direct effects of cadmium on intermediary metabolism in oysters have not been examined.

Coombs (1974) shows that 40% of Zn and Cu in *Ostrea edulis* is located in the low molecular weight soluble fraction of whole homogenates. The enzymes of glycolysis are found in the soluble intracellular fraction, and cadmium, a strong Zn and Cu antagonist, may prove to have a significant effect in this fraction.

7.3 MATERIALS AND METHODS

7.3.1 GENERAL

Oysters were kept in the standard flowing sea water system and at levels of cadmium of 0, 10 and 50 µg/l. For analysis, animals were transferred to Canberra in cool, moist conditions (approximately five hours), and maintained in 20 litre glass tanks (at one animal per litre) at the relevant cadmium level for seven days. Water was changed every second day. Bulk sea water was collected (approximately 200 litres) prior to each seven day period, but no attempt was made to

TABLE 7.1

SOME ENZYMES REQUIRING ZINC FOR ACTIVITY
(Dixon and Webb, 1964; McGilvery, 1970.)

Carboxylpeptidase A

Glutamate Dehydrogenase

Phosphoenolpyruvate carboxykinase (oyster) (Mustafa and Hochachka, 1973a)

Alcohol dehydrogenase

Numerous other enzymes are capable of being activated by a range of metals, including zinc and cadmium (Dixon and Webb, 1964)

maintain this water in a "fresh" condition. Aquaria were continuously bubbled with air.

Oysters described as "anaerobic" were removed from the aquaria 18 hours prior to analysis and left dry in the constant temperature room in which the aquaria were stored (20 $\pm$ 2^{o}C).

7.3.2 ASSAY OF METABOLITES

The organ to be examined was quickly excised and clamped between two aluminium blocks at the temperature of liquid nitrogen. The "snap frozen" tissue was stored under liquid nitrogen until all animals had been similarly processed (approximately 20 min). The frozen tissue was ground in a steel homogenizer under liquid nitrogen. Further preparation of the tissue and assay of the intermediates and nucleotides followed the methods of Behm and Bryant (1975). Oysters used in these experiments had been exposed to cadmium for between nine and 12 weeks.

7.3.3 ^{14}C GLUCOSE INCORPORATION

Oysters were kept as described above. Gills were removed from five animals per concentration, cut into pieces approximately 0.5 cm long and pooled for each cadmium concentration. Experimental vials were allocated at random to gill pieces within a cadmium concentration, and in the case of anaerobic gill pieces, 5% CO_2/N_2 bubbled sea water was used to hold the gills until preparation of all gills was complete. All gill pieces were then allowed to stand for 15 min under experimental conditions prior to the addition of radiocarbon. Deoxygenated sea water for the incubations was prepared by bubbling 500 ml of filtered (0.45 µm) sea water with 5% CO_2/N_2 in a Glove Bag (I^2R) which had been flushed with N_2 for 12 hours. Aerobic

sea water was prepared by bubbling sea water with air. In both cases pre-preparation pH was maintained.

Experimental vials were resealable, had a centre well containing 0.5 ml 2N KOH, and an outer well containing the tissue plus 4 ml of sea water at the relevant cadmium concentration. ^{14}C-U-glucose (4 µCi of 3 mCi/nmole) was added (carrier free) to the outer well to begin the incubation, and after the appropriate time at 20^{o}C, reactions were arrested by removing the tissue pieces, rinsing twice in sea water, once in distilled water, then transferring to 4 ml of 80% ethanol and storing at 0^{o}C until each piece could be homogenized. After homogenization in an all glass homogenizer, cell debris was removed by light centrifugation. The pellet was stored for later drying and weighing, while the supernatant was used for chromatography of the labelled intermediates. Aliquots of the 80% ethanol supernatant and the sea water incubation medium were counted by scintillation, and 500,000 cpm of each were subjected to two dimensional paper chromatography employing phenol/water and butanol/propionic acid systems, following the methods of Smith and Moses (1960). Chromatograms were applied to X-ray film (Kodirex medical X-ray film) for 14 days, and resulting radioactive spots were identified by co-chromatography techniques, by colour reaction using cadmium/ninhydrin dip (Atfield and Morris, 1961), and by elution and analysis by amino acid analyser. After identification spots were counted by cutting out and folding into the bottom of a scintillation vial and adding 10 ml PCS scintillant. $^{14}CO_2$ output was estimated by adding the contents of the centre well to 10 ml of PCS scintillant and counting.

Scintillation counting for all ^{14}C experiments was carried out in a Packard "Tri-Carb" counter, allowing at least 10,000 counts to accumulate, and recounting at 48 hour intervals until any indications of chemiluminesence were overcome. External counting standards were employed to indicate significant quenching of samples. Further dilutions were used when quenching was suspected.

7.3.4 $^{14}CO_2$ INCORPORATION

Oysters were exposed to cadmium for 16 weeks, but otherwise were as described for the experiment with glucose incorporation. A similar experimental system was employed here to that for glucose incorporation described above. The incubation volume of sea water was 3 ml and the number of animals was reduced to three per cadmium concentration. 3 µCi ^{14}C $NaHCO_3$ (50 µCi/ml) was added to the vials to commence the reaction, and the experiment was terminated as before, except that the tissue was rinsed once in distilled water only. One ml of the incubation sea water was added to 1 ml 2N HCl, evaporated and then counted by rehydrating with 1 ml water and adding 10 ml PCS scintillant. Further samples of the incubation sea water were chromatographed as previously described. Chromatograms were exposed to X-ray film for eight weeks.

7.3.5 STATISTICS

Routine statistical comparisons employed Students t test. Other tests, where used, are specifically noted.

7.4 RESULTS

Results of experiments involving incubation of gill pieces with $^{14}CO_2$ and ^{14}C labelled glucose are summarized in Tables 7.2, 7.3

and 7.6. Results of assays of pool sizes are presented in Tables 7.4 and 7.5.

7.4.1 ^{14}C LABELLED GLUCOSE

In both aerobic and anaerobic conditions levels of labelled glucose within the gill tissue were elevated by 10 µg/l cadmium, and depressed by 50 µg/l cadmium with respect to the controls. There was no significant difference between $^{14}CO_2$ production in the controls and either cadmium level, nor in levels of malate and asparate. Labelled glutamate levels appear to be enhanced by cadmium anaerobically.

Levels of labelled succinate were significantly depressed by 10 µg/l cadmium but not by 50 µg/l cadmium, in the aerobic situation. Levels of aerobically labelled alanine were significantly depressed by 50 µg/l cadmium, but raised by 10 µg/l cadmium. Labelled alanine was increased anaerobically by 10 µg/l cadmium.

7.4.2 $^{14}CO_2$ INCORPORATION

Single observations only are available from this experiment, because of the overall low incorporation of label into intermediates. The results, however, are internally consistent. For example, total label incorporated by the controls aerobically is similar to the total label incorporated anaerobically (which is to be expected following the hypothesis of simultaneous functioning of both pathways).

Features of Table 7.3 include the aerobic depression of labelled aspartate by cadmium at both 10 and 50 µg/l levels. A similar pattern was shown by glutamate and alanine aerobically. Both labelled succinate and labelled malate appeared to be elevated at 50 µg/l, although there was no change at 10 µg/l aerobically. The total amount of label incorporated aerobically into recoverable intermediates

TABLE 7.2(a)

Incorporation of ^{14}C from glucose into whole gill tissue during aerobic incubations. cpm/mg dry wt tissue, means ± one standard deviation of two observations. (-, not detected)

	Control				10 µg/l				10 µg/l			
hr	1	1.5	2	3	1	1.5	2	3	1	1.5	2	3
CO_2	156.6	1207.4	408.4	444.3	123.2	502.2	508.2	566.1	451.1	791.3	178	370.8
± lSD	±30.8	420.8	46.9	200.5	107.1	315.1	149.6	209.4	33.7	613.6	7.4	71.4
GLUCOSE	2048.5	2612.5	2384	1724.5	2221	2829	2973	3827*	827	1371	1574	1082*
± lSD	178.0	823.8	337.9	154.9	-	2534	36.1	2584	-	52	274	205
MALATE	-	87	103	152.5	50	85	60	52	22	72	106	147
± lSD	-	63.6	74.9	54.4	-	70.7	-	18.4	4	194	72	-
ASPARTATE	-	199	436	444	155	178	212	407	56	393	310	212
± lSD	-	73.5	-	79.2	-	58	-	151	22	293	61.5	123
SUCCINATE	289	4738	467	1701	272	1151	88	388*	110	354	4156	747
± lSD	-	-	318.2	657.6	-	117	62	68	4	-	3852	424
ALANINE	1361.5	4738	6567	6198	2256	4163	4691	11690	1671	3678	4809	2623*
± lSD	398.1	617.3	209.3	678.8	-	394	1583	5236	892	230	363	1429
GLUTAMATE	-	2.6	391	450.5	80	161	429	822	98	457	375	361
± lSD	-	67.9	210.7	112.4	-	-	40	394	52	163	77	295
TOTAL (-GLUCOSE)			30501				29089					22546

* Significantly different from the controls ($P \leq 0.05$, t test).

TABLE 7.2(b)

Incorporation of ^{14}C from glucose into whole gill tissue in anaerobic incubations. Means of two observations.

	Control				10 µg/l				50 µg/l			
hr	1	1.5	2	3	1	1.5	2	3	1	1.5	2	3
CO_2	53	82	266	369	344	57	267	322	167	234	301	320
GLUCOSE	1288	1555	1568	3314	2374	2137	2011	3905*	811	1724	1800	2026*
MALATE	150	162	230	213	116	138	77	304	95	115	132	345
ASPARTATE	253	289	538	970	218	418	240	1172	194	458	1002	352
SUCCINATE	1016	1336	2378	4158	812	1579	1369	4044	699	1200	1842	5752
ALANINE	1540	2295	2036	3423	3130	2671	4584	4179**	2123	2145	2166	3897
GLUTAMATE	73	68	113	262	93	122	240	406	62	129	537	252
TOTAL (- GLUCOSE)				22,273				26,902				24,519

* Significantly different from controls, $P \leq 0.05$ (t test)

** Significantly different from controls, $P \leq 0.05$ (2 way anova)

TABLE 7.3

Incorporation of $^{14}CO_2$ into gill tissue. cpm/mg dry wt tissue. Single observations.

AEROBIC INCUBATION	Control			10 µg/l			50 µg/l		
TIME OF INCUBATION (min)	15	30	180	15	30	180	15	30	180
ASPARTATE	110	238	1119	51	113	272	125	164	341
MALATE	23	54	43	54	44	49	47	33	81
GLUTAMATE	0	0	81	0	0	17	0	0	21
ALANINE	0	13	101	0	0	20	0	0	46
SUCCINATE/FUMARATE	0	0	81	0	0	90	0	0	351
TOTAL COUNTS	133	305	1425	105	157	448	172	197	840
ANAEROBIC INCUBATION									
ASPARTATE	0	60	286	0	33	385	31	85	91
MALATE	0	46	97	0	39	184	58	97	258
GLUTAMATE	0	0	18	0	0	0	0	0	23
ALANINE	0	0	53	0	0	85	0	0	53
SUCCINATE/FUMARATE	0	112	1003	0	99	1950	98	169	1914
TOTAL COUNTS	0	218	1457	0	171	2604	187	351	2339

TABLE 7.4

Levels of gill intermediates and nucleotides (nmol/g) in oysters maintained aerobically.

n = no. of observations a = no. of experiments

INTERMEDIATE	a	n	Control	10 µg/l	50 µg/l
			Means ± 1 standard deviation		
GLUCOSE	2	6	40.8 ± 50.7	54.6 ± 62.7	48.5 ± 22.2
F16DP	3	9	10.9 ± 6.8	11.7 ± 4.5	11.1 ± 4.8
DHAP	2	6	16.7 ± 8.3	21.3 ± 16.7	15.3 ± 8.2
G3P	2	6	14.7 ± 4.1	20.8 ± 11.3	17.8 ± 11.2
3PGA	3	9	109.2 ± 78.2	147.8 ± 81.4	97.1 ± 53.9
2PGA	3	9	32.3 ± 18.5	28.2 ± 16.9	16.11± 7.4*
PEP	3	9	31.4 ± 25.1	14.6 ± 11.0*	32.9 ± 24.3
PYRUVATE	3	9	21.1 ± 15.4	26.3 ± 12.6	25.7 ± 17.9
LACTATE	3	7	48.4 ± 31.7	77.6 ± 89.1	68.8 ± 65.9
MALATE	3	7	139.4 ± 22.1	100.0 ± 41.14*	132.1 ± 54.3
SUCCINATE	3	7	539.4 ± 312.0	527.0 ± 565.7	629.6 ± 387.0
ATP	3	9	196.7 ± 129.5	203.6 ± 113.8	196.0 ± 74.1
ADP	3	9	295.0 ± 108.6	365.7 ± 153.3	329.9 ± 136.0
AMP	3	9	231.1 ± 108.2	254.0 ± 71.1	157.8 ± 65.9
ENERGY CHARGE (Pooled)			0.48	0.47	0.53
ATP/ADP			0.66	0.56	0.59
TOTAL NUCLEOTIDES			6506	7410	6153

* Significantly different from controls at 0.05 level or less.

TABLE 7.5

Levels of gill intermediates and nucleotides (nmol/g) in oysters maintained anaerobically.

n = no. of observations a = no. of experiments

INTERMEDIATE	a	n	Control	10 µg/l	50 µg/l
GLUCOSE	1	3	29.3 ± 28.7	6.0 ± 6.0	6.6 ± 4.7
F16DP	2	6	8.7 ± 3.8	8.7 ± 4.3	6.0 ± 3.5
DHAP	1	3	8.7 ± 4.6	22.3 ± 21.3	10.0 ± 2.6
G3P	1	3	6.3 ± 0.6	4.0 ± 3.6	13.0 ± 7.9
3PGA	2	6	67.7 ± 27.7	57.0 ± 53.9	435.0 ± 459.1*
2PGA	2	6	14.8 ± 2.5	13.2 ± 4.2	20.7 ± 19.1
PEP	2	6	16.5 ± 6.3	15.8 ± 6.1	21.8 ± 18.4
PYRUVATE	2	6	21.8 ± 14.7	27.2 ± 21.1	11.2 ± 12.9
LACTATE	2	6	79.8 ± 74.3	191.5 ± 241.1	251.3 ± 197.5*
MALATE	2	6	93.0 ± 23.2	153.6 ± 65.5*	111.2 ± 51.9
SUCCINATE	2	6	2147.3 ±673	2115.3 ± 511.9	1105.8 ± 58.2*
ATP	2	6	144.8 ± 59.7	153.5 ± 108.5	82.7 ± 80.0
ADP	2	6	335.7 ± 12.1	416.0 ± 51.2	346.2 ± 159.8
AMP	2	6	271.8 ± 73.1	240.5 ± 73.6	251.3 ± 131.0
ENERGY CHARGE			0.42	0.45	0.38
ATP/ADP			0.43	0.37	0.33
TOTAL NUCLEOTIDES			4514	4860	4081

* Significantly different from controls at 0.05 level or less.

TABLE 7.6

Excretion of acid stable ^{14}C material by gill tissue following incubation with $^{14}CO_2$. cpm, single observations. C = control vial with no added tissue.

AEROBIC	15 min	30 min	180 min
C	69	73	69
Control	69	63	66
10 µg/l	68	67	67
50 µg/l	77	75	64

ANAEROBIC	15 min	30 min	180 min
C	77	72	71
Control	83	79	111
10 µg/l	71	70	107
50 µg/l	74	88	867*

* Significantly different from control ($P \leq 0.05$).

TABLE 7.7

ABBREVIATIONS

Enzymes, their reactants and products.

PEPCK : Phosphoenolpyruvate carboxykinase; phosphoenolpyruvate (PEP) and oxalacetate (OAA)

PK : Pyruvate kinase; PEP and pyruvate (PYR)

PDH : Pyruvate dehydrogenase; PYR and Acetyl Co-A

LDH : Lactic dehydrogenase; PYR and lactate

GPT : Glutamate - pyruvate transaminase; PYR and alanine

GOT : Glutamate - oxalacetate transaminase; OAA and aspartate

PC : Pyruvate carboxylase; PYR and OAA

FDPase : Fructose-1,6-diphosphatase; Fructose-1,6-diphosphate (Fl6DP) and fructose-6-phosphate (F6P)

G6Pase : Glucose-6-phosphatase; Glucose-6-phosphate (G6P) and glucose

"Malic enzyme" : PYR and OAA

Others :

AMP : Adenosine 5'-monophosphate

ADP : Adenosine 5'-diphosphate

ATP : Adenosine 5'-triphosphate

NAD : Nicotinamide-adenine dinucleotide (oxidized)

NADH : Nicotinamide-adenine dinucleotide (reduced)

GDH : Guanosine 5'-diphosphate

GTP : Guanosine 5'-triphosphate

αKG : Alpha ketoglutarate

DHAP : Dihydroxyacetone phosphate

G3P : Glyceraldehyde-3-phosphate

3PGA : 3-phosphoglyceric acid

2PGA : 2-phosphoglyceric acid

fum : Fumarate

succ : Succinate

mal : Malate

appeared to be depressed by cadmium.

Anaerobically, labelled aspartate, glutamate and alanine remained reasonably unaffected by cadmium. Labelled malate was clearly enhanced by 50 µg/l, and perhaps by 10 µg/l. Labelled succinate/fumarate was enhanced by cadmium at both concentrations. Total $^{14}CO_2$ incorporation was also elevated by both levels of cadmium.

Apart from one observation, incubation sea water showed no accumulation of label from $^{14}CO_2$ (Table 7.6). The anaerobic 50 µg/l incubation showed a level of acid stable ^{14}C significantly higher than all others, indicating some excretion of metabolites by the tissue. The compound(s) was not in sufficient quantity to enable identification.

7.4.3 POOL SIZES

Despite the very large standard deviations of the means, some pool sizes were significantly affected by cadmium. Aerobically (Table 7.4) AMP and 2PGA were depressed by 50 µg/l, and PEP and lactate were depressed by 10 µg/l.

Anaerobically, 3PGA and lactate were significantly enhanced by 50 µg/l while succinate was depressed. Malate and ADP were both enhanced by 10 µg/l cadmium.

Effects on nucleotide pool sizes were not statistically significant, apart from a depression of AMP by 50 µg/l aerobically, and an enhancement of ADP by 10 µg/l anaerobically. Energy charges, ATP/ADP ratios and total nucleotide pools were not significantly affected by cadmium treatment.

7.5 DISCUSSION

7.5.1 INTERMEDIARY METABOLISM

Evidence for the proposed sequence of steps in intermediary metabolism in the oyster gill has been previously discussed (Figure 7.1) and is supported by studies on ^{14}C labelled glucose and carbon dioxide incorporation into gill tissue presented in this thesis. Also, many of the intermediates of the reaction scheme have been demonstrated in measurable quantities.

Labelled CO_2 production from ^{14}C glucose was detected aerobically (Table 7.2), which supports the concept of a functional citric acid cycle in the gill, a primary site for aerobic CO_2 generation. Malate and succinate were labelled from ^{14}C glucose both aerobically and anaerobically, as were aspartate, alanine and glutamate. The experimental method did not allow detection of labelled pyruvate.

Fixation of $^{14}CO_2$ into succinate aerobically is slight, in contrast to the anaerobic situation, which indicates a reduction of flux of carbon to succinate via the "anaerobic" pathway, or a rapid oxidation of succinate, during aerobic conditions. Succinate produced anaerobically may accumulate as an end product, and after prolonged anaerobiosis can be observed as an excretory product (Hochachka and Mustafa, 1972). Hemolymph of oysters is known to contain high concentrations of many compounds, and it could well be possible that succinate produced anaerobically is transported and stored for later use in aerobic conditions.

The role of lactate in the gill is unknown, but anaerobically there is an elevation of pool size, and cadmium certainly has an effect

on this (to be described later). Lactate may be formed in response to the high NADH/NAD ratios present under anaerobic conditions. The reoxidation of NADH so achieved would permit the continuation of glycolysis (NAD requiring) if cadmium impaired PEPCK activity and the consequent regeneration of NAD at the level of OAA or FUM.

The pathways proposed are similar to that advanced by Hochachka and Mustafa (1972) and Hochachka, et. al. (1973), and fulfil the fundamental assumptions made by these authors concerning anaerobic metabolism in molluscs. Predictions about distribution of label into alanine and succinate, and maintainence of redox potentials can also be fulfilled under the proposed scheme.

7.5.2 EFFECT OF CADMIUM ON ENERGY PRODUCTION

The distribution of label from ^{14}C glucose and $^{14}CO_2$ is clearly altered by cadmium (Tables 7.2 and 7.2). The significance of these effects on energy production *in vivo* is unknown, but they demonstrate that there are effects of cadmium which can be readily measured using radiocarbon techniques.

An examination of the radiocarbon data reveals that cadmium probably has several different effects. First, aerobic metabolism appears to be inhibited by both 10 and 50 µg/l cadmium. Evidence for this conclusion is as follows:

(a) under aerobic conditions the amount of ^{14}C from $^{14}CO_2$ appearing in aspartate (probably via "malic enzyme") is reduced by cadmium (Table 7.3);

(b) aerobically labelled succinate from $^{14}CO_2$ is increased by 50 µg/l cadmium treatment, indicating an inhibition of aerobic utilization of succinate or a simple increase in the flux of carbon to succinate to

compensate for inhibition of the aerobic system.

Second, the anaerobic pathway glutamate → αKG → succinate may be inhibited by cadmium. This appears likely since:

(a) the amount of succinate labelled anaerobically from $^{14}CO_2$ is increased by cadmium (Table 7.3); (Note that succinate produced through the pathway glut. → αKG → succ. is not labelled by ^{14}C from either CO_2 or glucose.)

(b) the level of labelled succinate produced aerobically from $^{14}CO_2$ is increased by 50 µg/l cadmium treatment, but not by 10 µg/l. At the lower level of cadmium treatment the aerobic demand for succinate produced anaerobically may be less;

(c) alanine labelled aerobically from ^{14}C glucose is decreased by 50 µg/l cadmium treatment (Table 7.2).

It seems likely that the anaerobic pathway PEP → OAA → mal → succ. is not significantly inhibited by cadmium, since treatment with cadmium at both concentrations does not affect the levels of succinate labelled anaerobically from ^{14}C glucose.

In summary, the radiocarbon data indicates that the cadmium affected gill relies heavily on anaerobic pathways during aerobiosis, and that one of the anaerobic pathways may be partially inhibited. There may, alternatively, be an effect on the two transaminases, GPT and GOT, although preliminary experiments indicate that this is not the case.

A significant increase of ^{14}C in the incubation medium of the 50 µg/l anaerobic vial (Table 7.6) indicates excretion of labelled intermediates and, perhaps, impairment of a transport function of the gill. Tissue levels of labelled glucose (from supplied ^{14}C glucose, Table 7.2) are significantly depressed by cadmium, supporting the

possibility of cellular transport dysfunction in the gill, caused by 50 µg/l cadmium.

Treatment with 10 µg/l cadmium enhances the level of labelled glucose in gill tissue (Table 7.2). Such an enhancement may reflect an effect of cadmium on the transport processes of the gill, and is not inconsistent with the effects observed at 50 µg/l since many toxic compounds are known to exert a stimulatory effect at very low concentrations prior to inhibition at higher concentrations (hormology). (For a discussion of hormology see Luckey, Venugopal and Hutcheson, 1975.)

Cadmium appears to have no effect on the level of ^{14}C glucose derived label in succinate during anaerobiosis (Table 7.2). However, $^{14}CO_2$ incorporation into succinate doubles (Table 7.3), the malate pool size rises (Table 7.5), and $^{14}CO_2$ derived label in malate rises, indicating a likely increase in the anaerobic flow of carbon through one of the CO_2 fixing reactions. A simple interpretation of the situation is that there is a supply of endogenous non-glucose derived pyruvate to the system. Many amino acids are capable of supplying endogenous pyruvate.

Although the radiocarbon data shows that there are several probable effects of cadmium, and demonstrates the routes by which such effects are by-passed, probably a more instructive tool is that of assessing pool sizes of the intermediates. Enzymes with altered equilibrium positions *in vivo* can be localized by changes in the pool size of substrates or products. In examining pool sizes the net contribution from all possible avenues is assessed, avoiding the problems of randomization and dilution encountered when studying the

distribution of radioisotopes.

The pool size results (Tables 7.4 and 7.5) indicate that some of the enzymes involved in the metabolism of the triose phosphates (3PGA, 2PGA) may have their equilibrium positions altered by treatment with 50 µg/l cadmium. It is not possible to speculate precisely about effects of this magnitude on the normal functioning of the gill.

The significant effects of cadmium on anaerobic lactate production (Table 7.5) are important since lactate is not usually of great significance in the overall energy strategy of the gill. A further interpretation of this effect is not possible because of the paucity of knowledge concerning the normal role of lactate in the oyster gill.

An examination of the pool sizes of succinate shows (Tables 7.4 and 7.5) that this end product of the anaerobic pathways remains at normal levels despite treatment with cadmium, except after a period of anaerobiosis in the 50 µg/l treated group. The decrease in the succinate pool size under these conditions suggests:

(a) a decrease in succinate production. Such a possibility seems unlikely since there are no significant changes in the energy levels or in the level of ^{14}C labelling of the other end products, alanine and CO_2;

(b) an increase in the utilization of succinate. This possibility is unlikely in the absence of oxygen;

(c) excretion, either deliberately or through failure of membrane function. Results from the radiocarbon experiments (above) suggest the loss of a labelled metabolite to the incubation medium, so it seems probably that succinate is lost to the external environment during

anaerobiosis in response to a cadmium induced impairment of membrane function.

The effect of cadmium on the pool sizes of ATP, ADP and AMP was also assessed, and although a significant aerobic effect on AMP and an anaerobic effect on ADP was observed, and the total nucleotide pool may have dropped slightly, it is difficult to construct an overall perspective for the effects at this, the level of energy equivalents.

The absolute pool sizes of all the adenine nucleotides compare favourably with those reported by Zs-Nagy, et. al. (1972) for gill tissue of *Mytilus galloprovincialis*. That study reported ATP, ADP, and AMP to be 133, 372 and 230 nmol/g wet wt respectively, compared to 197, 295 and 231 nmol/g wet wt reported by this study. However, ATP/ADP ratios reported by Zs-Nagy ranged from 0.36 aerobically to 0.09 anaerobically, while the ATP/ADP ratios presented in this thesis range from 0.66 aerobically to 0.43 anaerobically. Some of the variation may be ascribed to experimental procedure, since the freeze clamping technique was not used by Zs-Nagy, although it is also possible that there are real differences between the two species.

It is possible that an effect of both 10 and 50 µg/l cadmium on glucose levels, alluded to briefly in previous pages, results from an effect on membrane transport. The simplest explanation is that cadmium affects the net rate of transport of glucose across the membrane, since there is no evidence to suggest a change in the rate of utilization of glucose, or in the size of the extracellular pools caused by cadmium. The effect may not be restricted to transport processes specific for glucose, since intracellular succinate levels are also affected by cadmium (Tables 7.5 and 7.6).

Similar effects of cadmium on membrane permeability have been reported from some vertebrates (e.g. Harkonen and Kormano, 1970), and it is possible that the membrane effects discussed here parallel those observed in vertebrates. Also the effects of cadmium on metabolism described by Harkonen and Kormano (1970) have certain similarities with the effects described in this chapter.

Whatever the mechanism resulting in the difference in labelled glucose levels within the gill tissue, the effect is potentially a limiting factor. It is well known that bivalves can absorb many dissolved substances across the gill surface (Bamford and Campbell, 1976). The gill of *C. commercialis* seems well adapted for this function, having a continuous surface of microvilli (*vide infra*). The true significance of this effect of cadmium may well await elucidation of the quantitative role such dissolved substances play in normal environmental conditions. *C. commercialis*, being primarily estuarine rather than fully marine, may be well situated to utilize this capability to the utmost.

Usuki (1962) has demonstrated that, in the oyster gill, glycogen is stored extracellularly, so any cellular glucose transport problems caused by cadmium may also be observed as an effective cellular starvation of the glucose produced from glycogen.

In summary, both 10 and 50 µg/l cadmium affects the levels of some intermediates of both aerobic and anaerobic pathways. Such effects are easily overcome by the cells during aerobic metabolism, since numerous avenues are open to by-pass the effects of cadmium and maintain energy levels.

The anaerobic pathway appears to be partially inhibited by 50 µg/l cadmium creating a further reduction in the diversity of strategies open to the cells for the supply of energy during anaerobiosis. The shift to anaerobiosis itself reduces the number of ways in which energy levels can be maintained, and the effect of cadmium is to further restrict the available energy producing options. It follows that it is during anaerobiosis that the effects of cadmium are the most hazardous to the gill.

There is, in addition, an effect of cadmium on the triose phosphates, and probably on the transport of glucose, the importance of which, in the experimental environmental conditions, remains to be assessed.

The gill tissue is apparently able to compensate for the effect of cadmium with ease, if the high energy phosphates can be taken as any indicator, and, in compensating, there appears to be a change of strategy to maintain levels of high energy phosphates and redox potentials within the cells.

The implications of this situation are:

first, under the experimental conditions employed for the exposure of the oysters to cadmium there is no significantly detrimental effect of cadmium on the overall energy status of the gill. However, the observations that during anaerobiosis *in vitro* the 50 µg/l treated group released an unidentified metabolite(s), failed to maintain the level of succinate pool, and glucose pools are either not maintained or are rapidly exchanging with the medium, may represent the effects of cadmium of the greatest potential hazard to the gill;

and second, the linking of amino acid metabolism to carbohydrate

metabolism to survive extended periods of anaerobiosis stresses the vital role of amino acids in energy metabolism. A possible interference by cadmium with the normal rate of utilization of amino acids could prove to be of crucial significance in situations of environmental stress (for example lowered salinity, high ambient temperatures and periods of prolonged anaerobiosis). Amino acids are used extensively in volume regulation in bivalves (Baginski and Pierce, 1975; Hammen, 1968) and both alanine and aspartate (together with taurine, proline and glycine) are thought to be the major amino acids involved in acclimation to salinity stress in bivalves (Hammen, 1968; Pierce, 1971). Any effect of cadmium on amino acid metabolism could also have effects other than alteration in energy status.

The different strategies adopted to overcome the few effects of cadmium shown in this thesis illustrate the great plasticity of the system for generation and maintainence of high energy phosphate bonds or their equivalents. It is evident that the early biochemical effects of cadmium are not liable to be evidenced by variations in high energy phosphate levels, but by some other parameters such as the shift in equilibrium position of some enzymes, a shift in emphasis from one pathway to another, or a change in flux of a critical pathway. These effects may be considered as truly sub-lethal effects.

7.6 PROSPECTS FOR FURTHER WORK.

Although the evidence presented indicates effects of cadmium on various metabolites, direct evidence will await demonstration of specific enzyme inhibition.

Since the quantitative flow of carbon through specific pathways may represent the earliest indicator of the effects of cadmium *in vivo*, it would be useful to assess the effects of cadmium on the flux of carbon through, say, the anaerobic pathway in conditions simulating a naturally stressful environment. Such experiments, which examine effects of cadmium at various salinities, temperatures, and nutritional regimes could well provide a more realistic assessment of the effects of cadmium on organisms exposed to normal environmental fluctuations.

The effects of cadmium on membrane function discussed in this chapter could be assessed by examining the composition of the pallial fluid following periods of anaerobiosis, assessing the levels of succinate, alanine and aspartate (and perhaps lactate).

CHAPTER 8

GENERAL DISCUSSION

Cadmium is widely distributed in the marine environment. Local areas, for example, near regions of industrial, urban or agricultural activities, contain levels of cadmium which are higher than natural background concentrations.

The solubility of cadmium in natural sea water is high, because of complexation with chlorides. The amount of cadmium in the soluble phase may be buffered against depletion by the insoluble pool (other than that absorbed by living organisms). Some metals form colloids or precipitates, allowing the soluble forms to be removed by physical processes (Duinker, 1975), but cadmium may be unique in its ability to remain in a largely soluble form in sea water under a wide variety of environmental conditions (Chapter 3).

The persistence of cadmium as the soluble form may present marine organisms with some unusual problems. Organisms, such as oysters, which filter large volumes of water, may be exposed to considerably more cadmium (on a g/g basis) than organisms having different feeding characteristics. The ability of plankton to accumulate cadmium from water, against a concentration gradient, is well known. The two possible routes for the accumulation of cadmium by oysters are first, directly from the soluble forms in water, and second, by ingestion of plankton and other particles. The feeding mechanism of the oyster maximizes the possibility of removing cadmium from both sources, and the oyster accumulates cadmium from either

source with similar efficiency (Chapter 5). It seems that the ability of oysters to accumulate large amounts of cadmium is attributable to three factors:

1. the oysters' feeding mechanism (filtering large volumes of water and ingesting small particles of organic material);
2. the ability of marine organisms to absorb and firmly bind cadmium (phytoplankton, bacteria, etc.); and
3. the solubility of cadmium in sea water (making much cadmium available for biological absorption).

The gill, the gut, the heart/kidney and the mantle contain the highest concentrations of cadmium at all water levels of cadmium tested (Chapter 4 and Chapter 5). The muscle and the gonad have lower levels of cadmium after exposure to cadmium in water. The distribution of cadmium within the oyster may be a guide to the sites of effect of cadmium. However, the effect which eventually limits the viability of the oyster may not necessarily be at a site of accumulation of cadmium.

The site of most cadmium absorption is the gut, although the cadmium need not necessarily be in food material. Since the gut always remains the major site of uptake, irrespective of the form of the cadmium challenge (i.e. soluble or particulate), it is likely that mucus produced by the palps or gill scavenges soluble cadmium from the water column which is then passed into the gut. When the oyster is feeding both the mucus and the food material may contain cadmium and make a major contribution to the total body load.

The absorption of cadmium from the gut is doubtless aided by the physico-chemical conditions of the gut, which may be capable of

releasing bound metal into solution. Particulate matter and other metals are transported across the gut wall by amoebocytes (Yonge, 1926) and their function in digestion may be one of the major factors controlling absorption of cadmium from the gut of the oyster. The presence of cadmium-laden amoebocytes in the gill (Chapter 5) and the rapid accumulation of cadmium by the kidney indicate that the blood plays a major role in the distribution of cadmium through the oyster. In addition to absorption through the gut, the observation that metal-laden cytosomes are present in the apical area of epithelial cells of the gill is evidence that cadmium is taken up directly from the water. This may form a second but quantitatively less important route of uptake for cadmium. The mantle may also be involved with the uptake of particles by phagocytic mechanisms into amoebocytes (Ruddell, 1971a).

The direction of net flow of cadmium through the gill is unknown. Metal-laden amoebocytes are not observed to migrate through the gill tissues, and metal-laden cytosomes do not appear to be transferred to the blood system (Chapter 5). Although a kinetic study was not attempted it is reasonable to assume that little cadmium is excreted across the gill surface since amoebocytes are only rarely shed through the gill (Stauber, 1950).

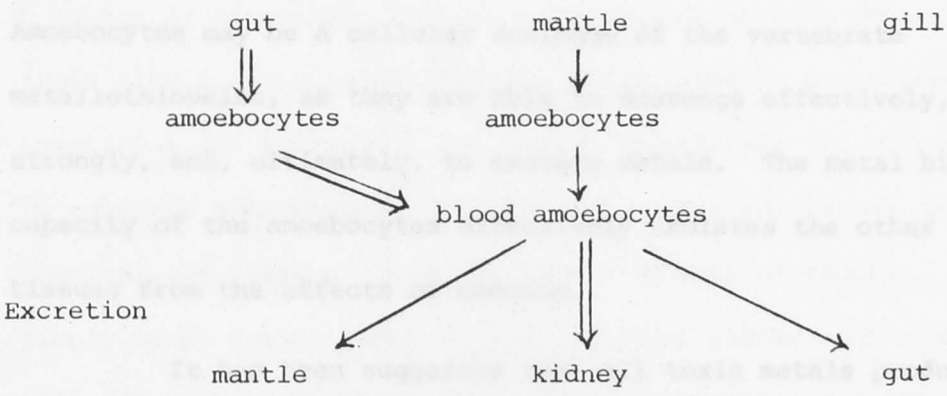
The major site of excretion is probably the kidney. The kidney is quickly labelled with cadmium after a challenge, and accumulates high concentrations of cadmium after a long term treatment (Chapters 4 and 5). The mechanism for the transfer of metal from amoebocytes to kidney, and from kidney to the external medium, is unknown. Amoebocytes excrete their contents into the gut lumen and across the mantle epithelium (Tripp, 1960) and these organs are

probably further sites of excretion of cadmium.

The accumulation and excretion of cadmium is shown, in diagrammatic form, in Figure 8.1. The model is a synthesis of data from this thesis and that obtained from the literature. A similar model has been independently proposed by George, Pirie and Coombs (1976) for the accumulation and excretion of iron by *Mytilus edulis*.

FIGURE 8.1

A model for the accumulation and excretion of cadmium by the oyster *C. commercialis*. The accumulating organs may retain some fraction of the absorbed cadmium.



Amoebocytes have long been known to play a role in defence against particulate foreign material, and they may also play a similar role in defence against soluble foreign material (Feng, 1967). It is apparent that the amoebocytes may transport cadmium through the tissues of the oyster. They may also play an important role in temporary detoxification of soluble cadmium. Amoebocytes are observed to contain large amounts of metal, while cells of surrounding tissues appear relatively free of cadmium (Chapter 5). Ruddell and Rains (1975) have

correlated the amount of zinc and copper in oysters with the total body content of basophilic amoebocytes. It is likely that such a relation also holds for cadmium in oysters. If this is true, any environmental factor influencing the number of amoebocytes in oysters could also alter the capacity of the oyster to accumulate and resist cadmium. An investigation of the kinetics of the production, circulation and loss of amoebocytes in oysters may be of significance in environmental management, and in the production of relatively metal-free oysters for human consumption.

The ability of oysters to tolerate high environmental levels of cadmium, and high internal levels of cadmium, may rest on the integrity of the amoebocytes and their capacity for cadmium binding. Amoebocytes may be a cellular analogue of the vertebrate metallothioneins, as they are able to scavenge effectively, bind strongly, and, ultimately, to excrete metals. The metal binding capacity of the amoebocytes effectively isolates the other cells and tissues from the effects of cadmium.

It has been suggested that all toxic metals produce an hormetic response, which may be defined as a stimulatory effect at low levels, prior to inhibition at higher levels (Luckey, Venugopal and Hutcheson, 1975). Some of the results in Chapter 7 suggest that this may be so for cadmium in oysters. There is no evidence available to suggest that the heightened response elicited by an hormetic level of toxicant confers a long term advantage on an organism, and indeed it is possible that it confers a disadvantage. It is possible that the relative toxicity of toxicants such as metals could be assessed using, as a standard reference point, the level of toxicant at which an

hormetic effect is first displayed (with statistical significance). That concentration of metal which just elicits such a response could represent the true boundary between the long term "safe" level and the level of "no effect". Assuming that the responses were assessed using organ functions after long term *in vivo* exposure to the toxicant, the level described above could be a much better estimate of the true "safe" level than extrapolation from acute toxicity tests.

No hormetic effects are evident in the results in Chapter 6; indeed, the lowest cadmium level tested (10 µg/l) resulted in a reduction in the filtration rate of particulate material. As a result, the long term safe level for oysters is likely to be below 10 µg/l, especially in areas where extremes of environmental variables occur. A number of sub-lethal effects were demonstrated in Chapter 7, although their significance is not clear. During average environmental conditions the ability of the oyster to maintain its energy levels, and to tolerate some membrane "leakiness", after sub-lethal challenge with cadmium, indicates a tolerance to the effects of cadmium. Again, it is likely to be during times of environmental stress that these sub-lethal effects cause significant detrimental physiological effects.

The maintenance of energy levels (Chapter 7) is expected, since a foremost cellular strategy is the preservation of energy levels and redox potentials. If energy levels are observed to decline, the effects of the toxicant may be approaching irreversibility with cellular malfunction imminent. During sub-lethal intoxication with cadmium, the oyster gill appears to rely on alternative pathways for energy production, which may be one of the mechanisms which allows oysters to tolerate high tissue levels of metals.

Although 10 µg/l cadmium may be a safe level for cadmium in drinking water, it is apparent that a similar level is not suitably "safe" for the long term success of oyster populations. Since oysters may not be the estuarine organisms most sensitive to cadmium, 10 µg/l may also be detrimental to others. The ability of oysters to accumulate large amounts of cadmium enables them to be used as qualitative indicators of polluted waters. In New South Wales it may be convenient to consider oysters with a body load of cadmium greater than that permitted in oysters for human consumption (10 µg/g [dry weight]) as originating from a cadmium polluted environment. The Statewide mean level of cadmium in oysters is approximately 1 µg/g (dry weight) (Mackay, et al., 1975), although oysters from leases near Sydney have a mean level of 5 µg/g (dry weight) cadmium. If the experimental results of Chapter 4 can be applied to natural populations of oysters with any certainty, a cadmium level of 10 µg/g in a mature oyster grown in the natural environment probably reflects a relatively low water level of cadmium.

APPENDIX 1

ANALYSIS OF CADMIUM LEVELS BY ATOMIC ABSORPTION SPECTROSCOPY

1. CARBON ROD ANALYZER

The carbon rod analyzer was employed to assess background cadmium levels in organs or when the total amount of cadmium likely to be present in the sample did not exceed 2 µg. At levels higher than 2 µg/sample flame spectroscopy was a more useful method.

Cadmium standards were prepared from a stock commercial preparation of cadmium metal in 0.1N HCl(BDH), and stored in polyethylene bottles which had previously been soaked for a minimum of one week in 5% nitric acid. Both samples and standards were prepared in 10% nitric acid (ARISTAR-BDH analytical grade).

Samples were dried at 80°C, ashed at 450°C in a closed nickel crucible, then taken up into nitric acid. Nickel did not interfere with the determination of cadmium. The ashing technique removed all traces of background absorbance, which, in nitric acid digests of oyster tissue, was capable of causing errors of 50% or greater. Nonetheless, background absorbance was periodically monitored (H_2 lamp).

Instrumentation: Varian Techtron AA5, CRA model 63 and chart recorder

Working conditions:	gasses	Inert (N_2) 6½
		H_2 1½
	lamp	3½ ma
Atomizer settings step cycle:	dry	20 sec @ 1½
	ash	15 sec @ 3
	atomize	2 sec @ 4

Peaks were clean, with no prior loss of metal, no after burn and a rapid return to base line conditions. No attempt was made to integrate the areas under the peaks, and peak heights were taken as simple measures of the absorbance. A minimum of five peaks were recorded from each sample. All samples were applied with an Oxford 1 µl micropipette, after soaking the tips in 10% nitric acid to remove contamination.

Day to day variations were noticed, but a typical relative standard deviation at 0.075 mg/l was 6.27%, calculated from 18 observations spread over a full day of analyses. To observe instrument drift a standard was assayed after every sixth test.

The working range of the carbon rod method was 0.001 mg/l to 0.4 mg/l, which is considerably higher than the recommended range. However it was an advantage to attempt to use a wide range to ensure sufficient overlap with the flame method. The standard curve, which was complex and could not be adequately described by any single simple expression, flattened off considerably after 0.2 mg/l. To increase accuracy a computer interpolation program "Spline" was used to interpolate the tests into the standard curve. The "Spline" library program fits a line, using a cubic function derived from four points,

to the two innermost points. The accuracy of the program was checked by graphical methods. A typical standard curve, with several unknowns interpolated using "Spline", is shown in Figure A1.1.

2. FLAME ATOMIC ABSORPTION SPECTROSCOPY

Instrumentation: EIL 240 AAS

EIL 236 digital readout

Test solutions were oyster ash in 2% nitric acid (diluted from the solutions described for carbon rod analysis). Standards (working range 0.1 mg/l to 2.5 mg/l) were prepared from the stock cadmium metal in 0.1 NHCl solution (1000 mg/l, BDH).

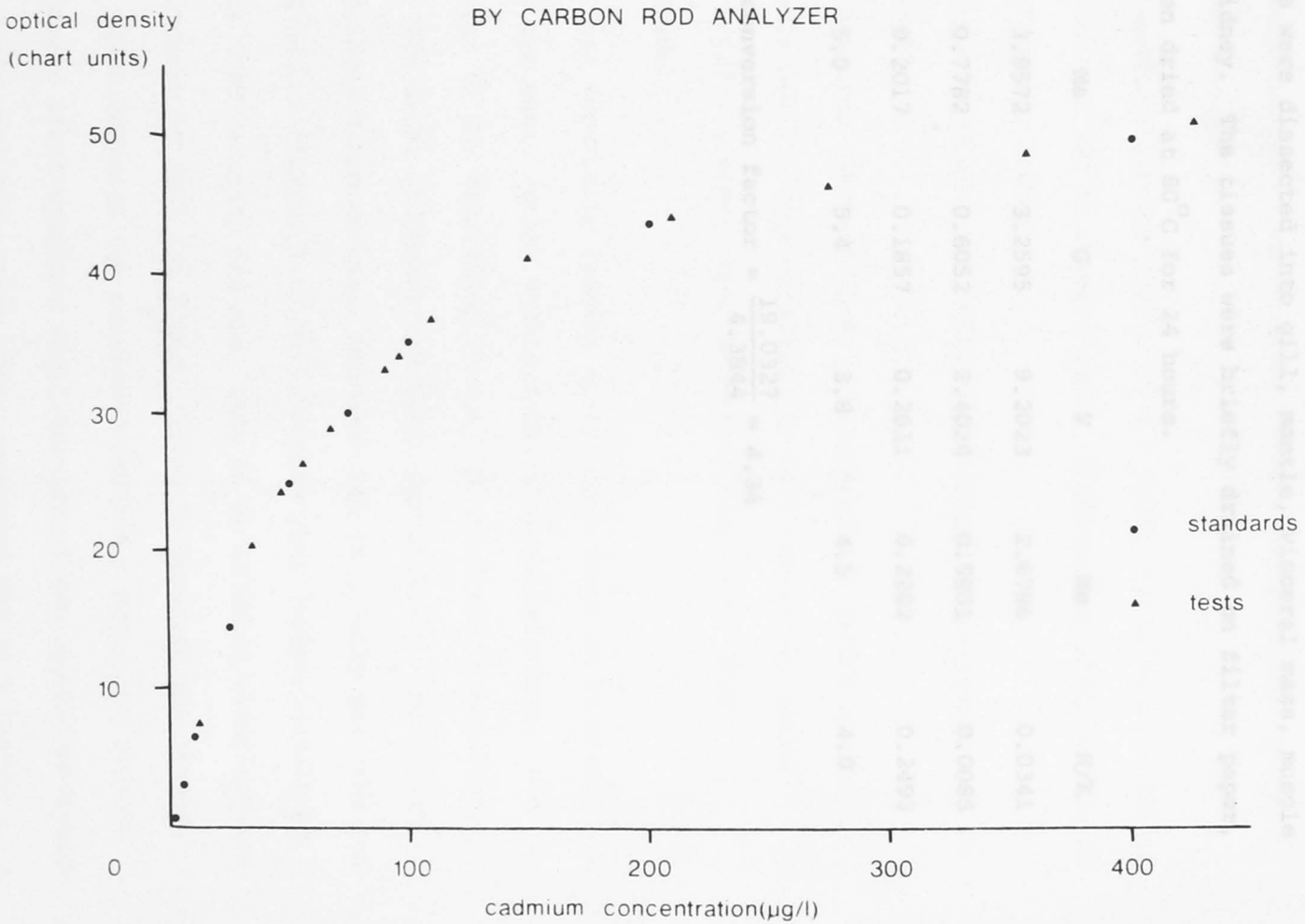
A 1.0 mg/l working standard solution was assayed by an independent laboratory (F. Darlington, Forestry Dept., SGS, ANU) and, in two independent tests gave values of 0.9961 and 0.9978 mg/l respectively.

Distilled water was aspirated between each test and standard, and the instrument adjusted to zero. Using this method precision is high, a typical RSD at 1.0 mg/l is 0.66%, calculated from seven determinations spread amongst the assay of 40 tests solutions. Although the standard curve is linear "Spline" was also used for interpolation of the unknowns.

FIGURE A1.1

A typical standard curve for the analysis of cadmium in oyster tissue samples using the carbon rod analyzer. The tests are interpolated using the "Spline" computer program.

FIGURE A1.1 STANDARD CURVE FOR ANALYSIS OF CADMIUM BY CARBON ROD ANALYZER



APPENDIX 2

CONVERSION FACTORS FOR DRY TO WET WEIGHTS

Five oysters held in the flowing sea water system at Cronulla for 24 weeks were dissected into gill, mantle, visceral mass, muscle and heart/kidney. The tissues were briefly drained on filter paper, weighed, then dried at 80°C for 24 hours.

	Ma	G	V	Ms	H/K
wet wt (g)	3.8572	3.2595	9.2023	2.6796	0.0341
dry wt (g)	0.7782	0.6052	2.4024	0.5901	0.0085
ratio	0.2017	0.1857	0.2611	0.2202	0.2493
factor	5.0	5.4	3.8	4.5	4.0

Whole body conversion factor = $\frac{19.0327}{4.3844} = 4.34$

APPENDIX 3

ASSAY OF CADMIUM IN SEA WATER

The technique used was one of solvent extraction, followed by flame atomic absorption spectroscopy (AAS), modified from Yeager, Cholak and Meiners (1973).

Materials: ammonium citrate buffer pH 8.5 (400g citric acid in 100 ml distilled water and 400 ml reagent grade ammonium hydroxide, adjust to pH 8.5, make up to 1 litre).

aqueous phenol red indicator solution (0.1% phenol red in distilled water).

ammonium tetramethylene dithiocarbamate (ATDC) solution made fresh each day (2% aqueous solution, BDH, laboratory grade)

N-butyl acetate (NBA) saturated with water (BDH, laboratory grade).

Procedure: Soak separating funnels and storage tubes in 5% nitric acid for at least one week. Rinse with distilled deionized water. Add 100 ml of water to the separating funnel, 10 ml of ammonium citrate buffer, and five drops of phenol red indicator solution. Mix. Add ammonium hydroxide dropwise until the solution is clearly past the end point of the colour change, i.e. until bright pink (approximately pH 9). Add 2 ml ATDC solution and mix. Add 10 ml of water saturated NBA and mix vigorously by hand for 2 min. Stand to separate the phases. The organic phase contains the complexed cadmium. Assay the organic phase for cadmium after complete equilibration of the atomic absorption spectrophotometer with NBA, using water saturated NBA as a blank,

Standards should be carried through the same process, including a background sample (filtered sea water only).

COMMENTS

The influence of suspended material on the recovery of cadmium from an unfiltered water sample is unknown. I have assumed that the effect is negligible. The cadmium-ATDC complex in NBA is stable for at least seven days, in the dark. Although the technique was found to give dubious results above 100 µg/l it may be applicable to concentrations below 1 µg/l using the carbon rod AAS technique. Samples thought to be near 100 µg/l were diluted by half (including standards) with distilled deionized water for assay. A typical standard curve is shown in Figure A3.1. Accuracy and precision of the technique are high. Four water samples were made up to 10 µg/l and extracted and assayed as described above. Duplicate observations were made on the samples, and the relative standard deviation (RSD) of the eight observations was 2.4%. Four water samples were prepared as above (10 µg/l), and, after one week standing in dark conditions at room temperature, displayed a mean of 9.2 µg/l cadmium (RSD = 1.9%).

Standard solutions were prepared from filtered sea water, but were otherwise as described in Appendix 1. The background level of cadmium in sea water used for the preparation of standards was ignored, and the blanks (filtered sea water) gave very low absorbances (0.01 O.D. units or less). Stripping the cadmium from sea water was not attempted.

Some sea water samples were directly assayed for cadmium using the carbon rod (flameless) method of AAS, but the results were unsatisfactory. The sea water matrix masked the cadmium signal, or the

metal was lost with the volatile components. The extraction of cadmium with the chelating agent described above frees the resulting solution from interference by the sea water matrix (non-atomic absorption) and achieves a ten-fold concentration of the metal in one step.

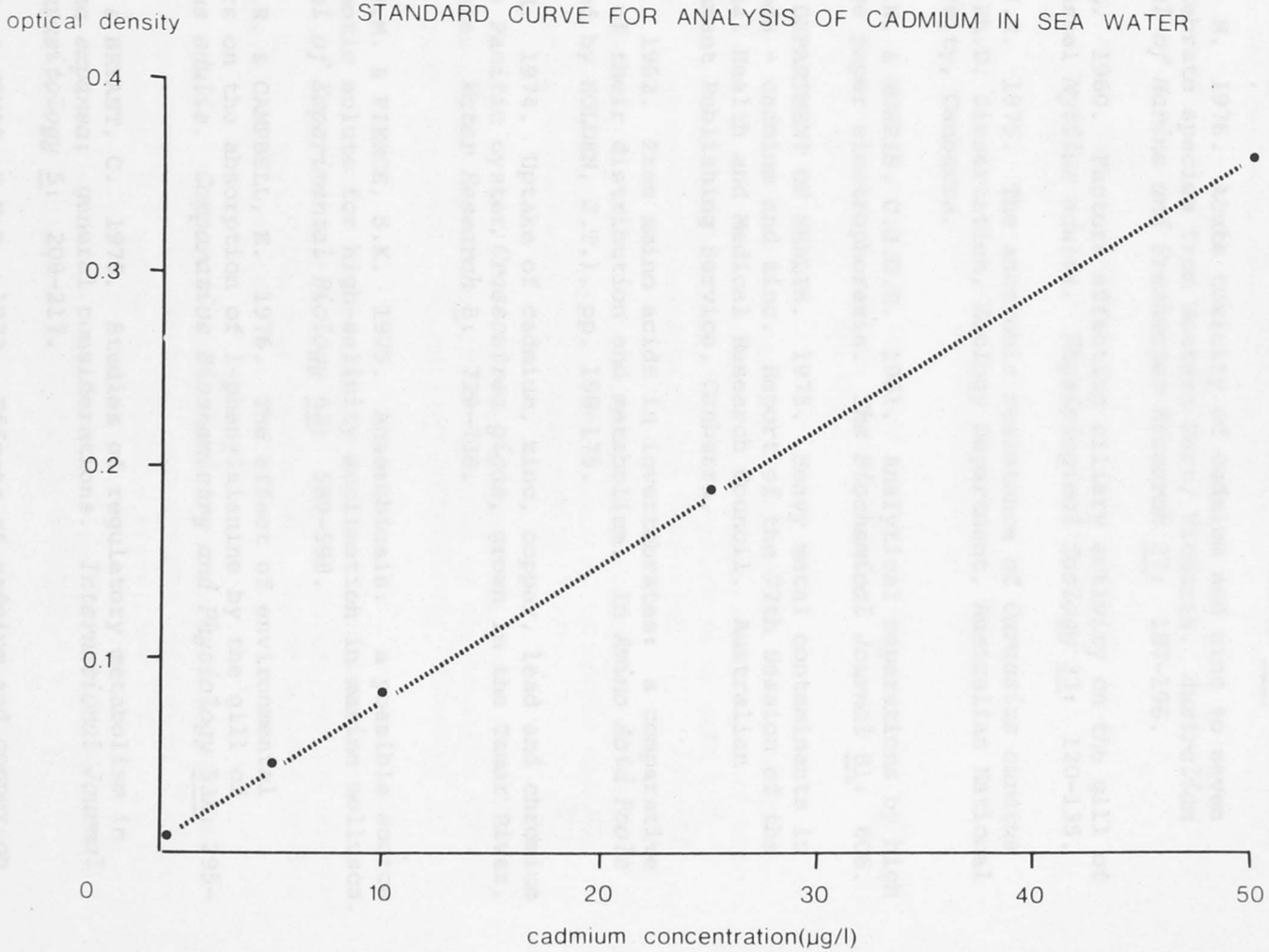
The technique described here could be applicable to other natural waters with an ionic strength less than that of sea water.

FIGURE A3.1

A standard curve prepared after solvent extraction of cadmium from sea water of known cadmium levels, and assay by flame AAS.

FIGURE A3.1

STANDARD CURVE FOR ANALYSIS OF CADMIUM IN SEA WATER



BIBLIOGRAPHY

ABDULLAH, M.I., ROYLE, L.G. & MORRIS, A.W. 1972. Heavy metal concentration in coastal waters. *Nature* (London) 235: 158-160.

AHSANULLAH, M. 1976. Acute toxicity of cadmium and zinc to seven invertebrate species from Western Port, Victoria. *Australian Journal of Marine and Freshwater Research* 27: 187-196.

AIELLO, E.L. 1960. Factors affecting ciliary activity on the gill of the mussel *Mytilus edulis*. *Physiological Zoology* 33: 120-135.

ANDERSON, J.R. 1975. The anaerobic resistance of *Carassius auratus* (L). Ph.D. dissertation, Zoology Department, Australian National University, Canberra.

ATFIELD, G.N. & MORRIS, C.J.O.R. 1961. Analytical separations by high voltage paper electrophoresis. *The Biochemical Journal* 81: 606.

AUSTRALIAN DEPARTMENT OF HEALTH. 1975. Heavy metal contaminants in seafoods - cadmium and zinc. Report of the 77th Session of the National Health and Medical Research Council. Australian Government Publishing Service, Canberra.

AWAPARA, J. 1962. Free amino acids in invertebrates: a comparative study of their distribution and metabolism. In *Amino Acid Pools* (edited by HOLDEN, J.T.), pp. 158-175.

AYLING, G.M. 1974. Uptake of cadmium, zinc, copper, lead and chromium in the Pacific oyster, *Crassostrea gigas*, grown in the Tamar River, Tasmania. *Water Research* 8: 729-738.

BAGINSKI, R.M. & PIERCE, S.K. 1975. Anaerobiosis: a possible source of osmotic solute for high-salinity acclimation in marine molluscs. *Journal of Experimental Biology* 62: 589-598.

BAMFORD, D.R. & CAMPBELL, E. 1976. The effect of environmental factors on the absorption of l-phenylalanine by the gill of *Mytilus edulis*. *Comparative Biochemistry and Physiology* 53A: 295-299.

BEHM, C.A. & BRYANT, C. 1975. Studies of regulatory metabolism in *Monieza expansa*: general considerations. *International Journal for Parasitology* 5: 209-217.

BILINSKI, E. & JONAS, R.E.E. 1973. Effects of cadmium and copper on the oxidation of lactate by rainbow trout (*Salmo gairdneri*) gills. *Journal of the Fisheries Research Board of Canada* 30: 1553-1558.

BROOKS, R.R. & RUMSBY, M.G. 1965. The biogeochemistry of trace element uptake by some New Zealand bivalves. *Limnology and Oceanography* 10: 521-527.

BROOKS, R.R. & RUMSBY, M.G. 1967. Studies on the uptake of cadmium by the oyster *Ostrea sinuata* (Lamarck). *Australian Journal of Marine and Freshwater Research* 15: 53-61.

BROWN, B. & AHSANULLAH, M. 1971. Effect of heavy metals on mortality and growth. *Marine Pollution Bulletin* 2(12): 182-187.

BROWN, V.M., SHAW, T.L. & SHURBEN, G.D. 1974. Aspects of water quality and the toxicity of copper to rainbow trout. *Water Research* 8: 797-803.

BROWN, V.M. 1968. The calculation of acute toxicity of mixtures of poisons to rainbow trout. *Water Research* 2: 723-733.

BRYAN, G.W. 1971. The effects of heavy metals (other than mercury) on marine and estuarine organisms. *Proceedings of the Royal Society of London, Series B* 177: 389-410.

CHAMBERS, J.E., McCORKLE, F.M., CARROLL, J.W., HEITZ, J.R., LEWIS, L. & YARBROUGH, J.E. 1975. Variation in enzyme activities of the American oyster, *Crassostrea virginica*, relative to size and season. *Comparative Biochemistry and Physiology* 51B: 145-150.

CHEN, C. & AWAPARA, J. 1969. Intracellular distribution of enzymes catalyzing succinate production from glucose in *Rangia* mantle. *Comparative Biochemistry and Physiology* 30: 727-737.

CHENG, T.C. & RUDO, B.M. 1976. Distribution of glycogen resulting from degradation of ^{14}C-labelled bacteria in the American oyster *Crassostrea virginica*. *Journal of Invertebrate Pathology* 27: 259-262.

CHRISTENSEN, G.M. 1975. Biochemical effects of methylmercuric chloride, cadmium chloride and lead nitrate on embryos and alevins of the brook trout *Salvelinus fontinalis*. *Toxicology and Applied Pharmacology* 32: 191-197.

CLOUD, P.E. 1965. Carbonate precipitation and dissolution in the marine environment. In *Chemical Oceanography* (edited by RILEY, J.P. & SKIRROW, G.) Volume 2, Academic Press, New York.

CONNOR, P.M. 1972. Acute toxicity of heavy metals to some marine larvae. *Marine Pollution Bulletin* 3: 190-192.

COOMBS, T.L. 1974. The nature of zinc and copper complexes in the oyster *Ostrea edulis*. *Marine Biology* 28: 1-10.

COUGHTRY, P.J. & MARTIN, M.H. 1976. The distribution of lead, zinc, cadmium and copper within the pulmonate mollusc *Helix aspersa* muller. *Ecologia* 23: 255-269.

CUNNINGHAM, P.A. & TRIPP, M.R. 1973. Accumulation and depuration of mercury in the American oyster *Crassostrea virginica*. *Marine Biology* 20: 14-19.

CUNNINGHAM, P.A. & TRIPP, M.R. 1975a. Accumulation, tissue distribution and elimination of $^{203}HgCl_2$ and $CH_3{}^{203}HgCl_2$ in the tissues of the American oyster *Crassostrea virginica*. *Marine Biology* 31: 321-334.

CUNNINGHAM, P.A. & TRIPP, M.R. 1975b. Factors affecting the accumulation and removal of mercury from tissues of the American oyster *Crassostrea virginica*. *Marine Biology* 31: 311-319.

DAVIES, P. 1973. Drugs affecting the golgi apparatus and lysozomes. In *Fundamentals of Cell Pharmacology* (edited by Dikstein, S.), Thomas Books.

DAVIS, B.D., DULBECCO, R., EISEN, H.N., GINSBERG, H.W. & WOOD, W.B. 1967. *Microbiology*, Harper and Row, New York.

DIXON, M. & WEBB, E.C. 1964. *Enzymes*, Second Edition, Longman, London.

DOOLAN, K.J. & SMYTH, L.E. 1973. Cadmium content of some New South Wales waters. *Search* 4(5): 162-163.

DUINKER, J.C. 1975. Mobilization of metals in the Dutch Wadden Sea. In *Ecological Toxicology Research* (edited by McINTYRE, A.D. & MILLS, C.F.), p. 167, Plenum Press, New York.

EISLER, R., ZAROOGIAN, G.E. & HENNEKEY, R.J. 1972. Cadmium uptake by marine organisms. *Journal of the Fisheries Research Board of Canada* 29: 1367-1369.

ENGEL, H.R. & NEAT, M.J. 1970. Glycolytic and gluconeogenic enzymes in the quahog *Mercenaria mercenaria*. *Comparative Biochemistry and Physiology* 37: 397-403.

ERNST, R., ALLEN, H.E. & MANCY, K.H. 1975. Characterization of trace metal species and measurement of trace metal stability constants by electrochemical techniques. *Water Research* 9: 969-979.

FENG, S.Y. 1967. Response of molluscs to foreign bodies, with special reference to the oyster. *Federation Proceedings* 26: 1685-1692.

FLORENCE, T.M. 1972. Determination of trace metals in marine samples by anodic stripping voltammetry. *Electroanalytical Chemistry and Interfacial Electrochemistry* 35: 237-245.

FOWLER, S.W. & BENAYOUN, G. 1974. Experimental studies on cadmium flux through marine biota. In *Comparative Studies of Food and Environmental Contamination*, pp. 159-189, IAEA-SM-175/10, Vienna.

FOWLER, B.A., WOLFE, D.A. & HETTLER, W.F. 1975. Mercury and iron uptake by cytosomes in mantle epithelial cells of quahog clams, *Mercenaria mercenaria*, exposed to mercury. *Journal of the Fisheries Research Board of Canada* 32: 1767-1775.

FRIBERG, L., PISCATOR, M., NORDBERG, G.F. & KJELLSTROM, T. 1974. *Cadmium in the Environment*, CRC Press, Cleveland, Ohio.

GALTSOFF, P.S. 1953. Accumulation of manganese, iron, copper and zinc in the body of the American oyster *Crassostrea virginica*. *Anatomical Record* 117: 601-602 (abstract).

GALTSOFF, P.S. 1964. The American oyster *Crassostrea virginica* gmelin. *Fishery Bulletin of the Fish and Wildlife Service, U.S. Department of the Interior*, Volume 64, U.S. Government Printing Office, Washington.

GARDINER, J. 1974a. The chemistry of cadmium in natural water - I: A study of cadmium complex formation using the cadmium specific-ion electrode. *Water Research* 8: 23-30.

GARDINER, J. 1974b. The chemistry of cadmium in natural water - II: The adsorption of cadmium on river muds and naturally occurring solids. *Water Research* 8: 157-164.

GEORGE, S.G., PIRIE, B.J.S. & COOMBS, T.L. 1976. The kinetics of accumulation and excretion of ferric hydroxide in *Mytilus edulis* (L) and its distribution in the tissues. *Journal of Experimental Marine Biology and Ecology* 23: 71-84.

GOLDBERG, E.D. 1965. Minor elements in sea water. In *Chemical Oceanography* (edited by RILEY, J.P. & SKIRROW, G.), p. 163, Academic Press, New York.

GOULD, E. & KAROLUS, J. 1974. Physiological response of the cunner *Tautogolabrus adspersus* to cadmium. V: Observations on the biochemistry. *U.S. Department of Commerce, National Oceanic and Atmospheric Administration Technical Report* NMFS SSRF-681, p. 21, U.S. Department of Commerce Publication.

GRAY, J.S. 1974. Synergistic effects of three heavy metals on growth rates of a marine ciliate protozoan. In *Pollution and Physiology of Marine Organisms* (edited by VERNBERG, F.J. & VERNBERG, W.B.), p. 465.

GRAY, J.S. 1976. Are marine baseline surveys worthwhile? *New Scientist*, 29 April, 1976, 219-221.

HAMMEN, C.S. 1968. Aminotransferase activities and amino acid excretion of bivalve molluscs and brachipods. *Comparative Biochemistry and Physiology* 26: 697-705.

HAMMEN, C.S. 1969. Metabolism of the oyster *Crassostrea virginica*. *American Zoologist* 9: 309.318.

HAMMEN, C.S. & LUM, S.C. 1966. Fumarate reductase and succinate dehydrogenase activities in bivalve molluscs and brachipods. *Comparative Biochemistry and Physiology* 19: 775-781.

HAUG, F.M.S. 1967. Electron microscopical localization of zinc in hippocampal mossy fibre synapses by a modified sulfide-silver technique. *Histochemie* 8: 355-368.

HARKONEN, M. & KORMANO, M. 1970. Acute cadmium-induced changes in the energy metabolism of the rat testis. *Journal of Reproduction and Fertility* 21: 221-226.

HAVEN, D.S. & MORALES-ALAMO, R. 1970. Filtration of particles from suspension by the American oyster *Crassostrea virginica*. *Biological Bulletin* 139: 248-264.

HELZ, G.R., HUGGETT, R.J. & HILL, J.M. 1975. Behaviour of Mn, Fe, Cu, Zn, Cd and Pb discharged from a wastewater treatment plant into an estuarine environment. *Water Research* 9: 631-636.

HEM, J.D. 1972. Chemistry and occurrence of cadmium and zinc in surface water and groundwater. *Water Resources Research* 8: 661-679.

HESS, C.T., SMITH, C.W. & PRICE, A.H. 1975. Model for the accumulation of radionuclides in oysters and sediments. *Nature* (London) 258: 225-226.

HILTIBRAN, R.C. 1971. Effects of cadmium, zinc, manganese and calcium on oxygen and phosphate metabolism of bluegill liver mitochondria. *Journal of the Water Pollution Control Federation* 43: 818-823.

HOCHACHKA, P.W. 1975. An exploration of metabolic and enzyme mechanisms underlying animal life without oxygen. In *Biochemical and Biophysical Perspectives in Marine Biology*, Volume 2 (edited by MALINS, D.C. & SARGENT, J.R.), pp. 107-136, Academic Press, New York.

HOCHACHKA, P.W. & MUSTAFA, T. 1972. Invertebrate facultative anaerobiosis. *Science* (N.Y.) 178: 1056-1060.

HOCHACHKA, P.W. & MUSTAFA, T. 1973. Enzymes in facultative anaerobiosis of molluscs - I. Malic enzyme of oyster adductor muscle. *Comparative Biochemistry and Physiology* 45B: 625-637.

HOCHACHKA, P.W. & SOMERO, G.N. 1973. *Strategies of Biochemical Adaptation*, W.B. Saunders, London.

HOCHACHKA, P.W., FIELDS, J. & MUSTAFA, T. 1973. Animal life without oxygen: basic biochemical mechanisms. *American Zoologist* 13: 543-555.

HORVATH, G.J., HARRISS, R.C. & MATTRAW, H.C. 1972. Land development and heavy metal distribution in the Florida Everglades. *Marine Pollution Bulletin* 3: 182-183.

HUGGETT, R.J., BENDER, M.E. & SLONE, H.D. 1973. Utilizing metal concentration relationships in the eastern oyster, *Crassostrea virginica*, to detect heavy metal pollution. *Water Research* 7: 451-460.

HUMPHREY, G.F. 1950. Glycolysis in oyster muscle. *Australian Journal of Experimental Biology and Medical Science* 28: 151-160.

HUTCHESON, M.S. 1974. The effect of temperature and salinity on cadmium uptake by the blue crab *Callinectes sapidus*. *Chesapeake Science* 15: 237-241.

JAAKKOLA, T., TAKAHASHI, H., SOININEN, R., RISSANEN, K., & MIETTINEN, J.K. 1972. Cadmium content of sea water, bottom sediment and fish, and its elimination rate in fish. In *Radiotracer Studies of Chemical Residues in Food and Agriculture*, IAEA-PL-469/7, Vienna.

JACKIM, E., HAMLIN, J.M. & SONIS, S. 1970. Effects of metal poisoning on five liver enzymes in the killifish *Fundulus heteroclitus*. *Journal of the Fisheries Research Board of Canada* 27: 383-390.

JODREY, L.H. & WILBUR, K.M. 1955. Studies on shell formation. IV. The respiratory metabolism of the oyster mantle. *Biological Bulletin* 108: 346-358.

JONES, M.B. 1973. Influence of salinity and temperature on the toxicity of mercury to marine and brackish water isopods (Crustacea). *Estuarine and Coastal Marine Science* 1: 425-431.

JORGENSEN, C.B. 1955. Quantitative aspects of filter feeding in invertebrates. *Biological Reviews* 30: 391-454.

KNAUER, G.A. & MARTIN, J.H. 1973. Seasonal variations of cadmium, copper, manganese, lead and zinc in water and phytoplankton in Monterey Bay, California. *Limnology and Oceanography* 18(4): 597-604.

KOPLER, F.C. & MAYER, J. 1973. Concentrations of five trace metals in the waters and oysters, *Crassostrea virginica*, of Mobile Bay, Alabama. *Proceedings of the National Shellfisheries Association* 63: 27-34.

LIVINGSTONE, D.R. & BAYNE, B.L. 1974. Pyruvate kinase from the mantle tissue of *Mytilus edulis* L. *Comparative Biochemistry and Physiology* 48B: 481-497.

LONGWELL, A.C. 1976. Review of genetic and related studies on commercial oysters and other pelecypod molluscs. *Journal of the Fisheries Research Board of Canada* 33: 1100-1107.

LOVETT, R.J., GUTENMANN, W.H., PAKKALA, I.S., YOUNGS, W.D., LISK, D.J., BURDICK, G.E. & HARRIS, E.J. 1972. A survey of the total cadmium content of 406 fish from 49 New York State fresh waters. *Journal of the Fisheries Research Board of Canada* 29: 1283-1290.

LUCKEY, T.D., VENUGOPAL, B. & HUTCHESON, D. 1975. Heavy metal toxicity, safety and hormology. *Supplement to Environmental Quality and Safety*, Volume 1 (edited by COULSTON, F., & KORTE, F.), G. Thieme, Stuttgart.

MACKAY, N.J., WILLIAMS, R.J., KACPRZAC, J.L., KAZACOS, M.N., COLLINS, A.J. & AUTY, E.H. Heavy metals in cultivated oysters, *Crassostrea commercialis=Saccostrea cucullata*, from the estuaries of New South Wales. *Australian Journal of Marine and Freshwater Research* 26: 31-46.

MALANGA, C.J. & AIELLO, E.L. 1972. Succinate metabolism in the gills of the mussels *Modiolus demissus and Mytilus edulis*. *Comparative Biochemistry and Physiology* 43B: 795-806.

McGILVERY, R.W. 1970. *Biochemistry. A Functional Approach.* W.B. Saunders, London.

MERALI, Z., KACEW, S., & SINGHAL, R.L. 1974. Alterations to rat liver and kidney cortex metabolism after acute and chronic exposure to cadmium (abstract). *Toxicology and Applied Pharmacology* 29(1): 140.

MERRILL, A.S. & BOSS, K.J. 1966. Benthic ecology and faunal change relating to oysters from a deep basin in the lower Patuxent River, Maryland. *Proceedings of the National Shellfisheries Association* 56: 81-87.

MUSTAFA, T. & HOCHACHKA, P.W. 1973a. Enzymes in facultative anaerobiosis of molluscs - II. Basic catalytic properties of phosphoenolpyruvate carboxykinase in oyster adductor muscle. *Comparative Biochemistry and Physiology* 45B: 639-655.

MUSTAFA, T. & HOCHACHKA, P.W. 1973b. Enzymes in facultative anaerobiosis of molluscs - III. Phosphoenolpyruvate carboxykinase and its role in aerobic-anaerobic transition. *Comparative Biochemistry and Physiology* 45B: 657-667.

NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL. 1972. Appendix IX. Report of the 75th Session of Council, November, Canberra.

NELSON, T.C. 1960. The feeding mechanism of the oyster - II. On the gills and palps of *Ostrea edulis, Crassostrea virginica* and *C. angulata*. *Journal of Morphology* 107: 163-204.

NEW SOUTH WALES CLEAN WATERS ACT AND REGULATIONS, 1970. Effective on 3 November, 1972. *N.S.W. Government Gazette* 117: November, 1972.

NIELSEN, S.A. 1974. Vertical concentration gradients of heavy metals in cultured mussels. *New Zealand Journal of Marine and Freshwater Research* 8(4): 631-636.

NIELSEN, S.A. & NATHAN, A. 1975. Heavy metal levels in New Zealand molluscs. *New Zealand Journal of Marine and Freshwater Research* 9(4): 467-481.

NORDBERG, G.F. 1972. Cadmium metabolism and toxicity. *Environmental Physiology and Biochemistry* 2: 7-36.

O'HARA, J. 1973. Cadmium uptake by fiddler crabs exposed to temperature and salinity stress. *Journal of the Fisheries Research Board of Canada* 30: 846-848.

OLAFSON, R.W. & THOMPSON, J.A.J. 1974. Isolation of heavy metal binding proteins from marine vertebrates. *Marine Biology* 28: 83-86.

OLSON, K.R. & HARRELL, R.C. 1973. Effect of salinity on acute toxicity of mercury, copper and chromium for *Rangia cuneata* (Pelecypoda, Mactridae). *Contributions in Marine Science*, Texas University 17: 9-13.

OWEN, G. 1966. Feeding. In *Physiology of Mollusca* (edited by WILBUR, K.M. & YONGE, C.M.), Volume II, Academic Press, New York.

PEDEN, J.D., CROTHERS, J.H., WATERFALL, C.E. & BEASLEY, J. 1973. Heavy metals in Somerset marine organisms. *Marine Pollution Bulletin* 4(1): 7-9.

PENTREATH, R.J. 1973. The accumulation from water of ^{65}Zn, ^{54}Mn, ^{58}Cd, and ^{59}Fe by the mussel, *Mytilus edulis*. *The Journal of the Marine Biological Association* (U.K.) 53: 127-143.

PERRIN, D.D. 1975. The selection of masking agents for use in analytical chemistry. In *CRC Critical Reviews in Analytical Chemistry*, Volume 6.

PERRIN, D.D. & DEMPSEY, B. 1974. *Buffers for pH and Metal Ion Control*, Chapman and Hall, London.

PIERCE, S.K. 1971. A source of solute for volume regulation in marine mussels. *Comparative Biochemistry and Physiology* 38A: 619-635.

PIHL, E. 1967. Ultrastructural localization of heavy metals by a modified sulfide-silver method. *Histochemie* 10: 126-139.

PIHL, E. & FALKMER, S. Trials to modify the sulfide-silver method for ultrastructural tissue localization of heavy metals. *Acta Histochemica* (Jena) 27: 34-41.

POPHAM, J.D. & WEBSTER, W.S. 1976. The ultrastructural localization of cadmium. *Histochemistry* 46: 249-259.

PRESTON, A. 1973. Cadmium in the marine environment of the United Kingdom. *Marine Pollution Bulletin* 4(7): 105-107.

PRINGLE, B.H. HISSONG, D.E., KATZ, E.L. & MULAWKA, S.T. 1968. Trace metal accumulation by estuarine molluscs. *Journal of the Sanitary Engineering Division, Proceedings of the American Society of Civil Engineers* 94: 455-475.

RATKOWSKY, D.A., THROWER, S.J., EUSTACE, I.J. & OLLEY, J. 1974. A numerical study of the concentration of some heavy metals in Tasmanian oysters. *Journal of the Fisheries Research Board of Canada* 31: 1165-1171.

ROMERIL, M.G. 1971. The uptake and distribution of ^{65}Zn in oysters. *Marine Biology* 9: 347-354.

RUDDELL, C.L. 1971a. The fine structure of oyster agranular amoebocytes from regenerating wounds in the pacific oyster *Crassostrea gigas*. *Journal of Invertebrate Pathology* 18: 260-268.

RUDDELL, C.L. 1971b. The fine structure of granular amoebocytes of the Pacific oyster *Crassostrea gigas*. *Journal of Invertebrate Pathology* 18: 269-275.

RUDDELL, C.L. & RAINS, D.W. 1975. The relationship between zinc, copper and the basophils of two Crassostreid oysters, *C. gigas* and *C. virginica*. *Comparative Biochemistry and Physiology* 51A: 585-591.

SCHMIDT, R. & SCHULTKA, R. 1974. Präparationstechnische voraussetzungen zum kombinierten Licht- und Elektronenoptischen nachweis von schwermetallen. *Acta Histochemica* (Jena) 50: 212-221.

SCHULZ-BALDES, M. 1974. Lead uptake from sea water and food, and lead loss in the common mussel *Mytilus edulis*. *Marine Biology* 25: 177-194.

SEGAR, D.A., COLLINS, J.D. & RILEY, J.P. 1971. The distribution of the major and some minor elements in marine animals. II. Molluscs. *Journal of the Marine Biological Association* (U.K.) 51: 131-136.

SHAW, W.N. 1963. Index of condition and percent solids of raft grown oysters in Massachusetts. *Proceedings of the National Shellfisheries Association* 52: 47-52.

SHAW, T.L. & BROWN, V.M. 1974. The toxicity of some forms of copper to rainbow trout. *Water Research* 8: 377-382.

SHUSTER, C.N. & PRINGLE, B.H. 1969. Trace metal accumulation by the American eastern oyster *Crassostrea virginica*. *Proceedings of the National Shellfisheries Association* 59: 91-103.

SIMPSON, J.W. & AWAPARA, J. 1964. Phosphoenolpyruvate carboxykinase activity in invertebrates. *Comparative Biochemistry and Physiology* 12: 457-464.

SIMPSON, J.W. & AWAPARA, J. 1965. Biosynthesis of glucose from pyruvate-2-^{14}C and aspartate-3-^{14}C in a mollusc. *Comparative Biochemistry and Physiology* 15: 1-6.

SIMPSON, J.W. & AWAPARA, J. 1966. The pathways of glucose degradation in some invertebrates. *Comparative Biochemistry and Physiology* 18: 537-548.

SMITH, A.E. 1973. A study of the variation with pH of the solubility and stability of some metal ions at low concentrations in aqueous solution. Part 1. *Analyst* 98: 65-68.

SMITH, M.J.H. & MOSES, V. 1960. Effects of salicylate and 2:4-dinitrophenol on the incorporation of ^{14}C from labelled glucose and acetate into the soluble intermediates of isolated rat tissues. *The Biochemical Journal* 76: 579-584.

SOKAL, R.R. & ROHLF, F.J. 1969. *Biometry. The Principles and Practice of Statistics in Biological Research*, W.H. Freeman, San Francisco.

SPENCER, D.W. & SACHS, P.L. 1970. Some aspects of the distribution, chemistry and mineralogy of suspended matter in the gulf of Maine. *Marine Geology* 9(2(: 117-136.

SPORN, A., DINU, I. & STOENESCU, L. 1970. Influence of cadmium administration on carbohydrate and cellular energetic metabolism in the rat liver. *Revue Roumaine de Biochimie* 7(4): 299-305.

SPRAGUE, J.B. 1969. Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. *Water Research* 3:793-821.

SPRAGUE, J.B. 1970. Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. *Water Research* 4: 3-32.

SPRAGUE, J.B. 1971. Measurement of pollutant toxicity to fish. III. Sublethal effects and "safe" concentrations. *Water Research* 5: 245-266.

STAUBER, L.A. 1950. The fate of India ink injected intracardially into the oyster *Ostrea virginica* gmelin. *Biological Bulletin* 98: 227-241.

STENZEL, H.B. 1971. Oysters. In *Treatise on Invertebrate Paleontology*, Part N, Volume 3, pp. N953-N1224, Geological Society of America.

STRUEMPLER, W. 1973. Adsorption characteristics of silver, lead, cadmium, zinc and nickel on borosilicate glass, polyethylene and polypropylene container surfaces. *Analytical Chemistry* 45: 2251.

TAKATSUKI, S. 1934. On the nature and function of the amoebocytes of *Ostrea edulis*. *Quarterly Journal of the Microscopical Society* 76: 379-431.

TASK GROUP ON METAL ACCUMULATION. 1973. Accumulation of toxic metals with special reference to their absorption, excretion and biological half-times. *Environmental Physiology and Biochemistry* 3: 65-107.

THORP, V.J. & LAKE, P.S. 1974. Toxicity bioassays of cadmium on selected freshwater invertebrates and the interaction of cadmium and zinc on the freshwater shrimp *Paratya tasmaniensis* riek. *Australian Journal of Marine and Freshwater Research* 25: 97-104.

THROWER, S.J. & EUSTACE, I.J. 1973. Heavy metals in Tasmanian oysters in 1972. *Australian Fisheries*, October, 1973: 7-10.

TRIPP, M.R. 1960. Mechanisms of removal of injected microorganisms from the American oyster *Crassostrea virginica* gmelin. *Biological Bulletin* 119: 210-223.

TRIPP, M.R. 1963. Cellular responses of molluscs. *Annals of the New York Academy of Sciences* 113: 467-474.

USUKI, I. & OKAMURA, N. 1956. Glycolytic intermediates in the oyster gills. *Tohoku University Science Reports, Fourth Series, Biology* 22: 225-232.

USUKI, I. 1962a. Distribution of glycogen, fat and phospholipid in the gill tissues of certain marine bivalves. *Tohoku University Science Reports, Fourth Series, Biology* 28: 53-57.

USUKI, I. 1962b. Energy source for the ciliary movement and the respiration and its metabolism in oyster gill. *Tohoku University Science Reports, Fourth Series, Biology* 28: 59-83.

USUKI, I. 1962c. The stoppage and the recovery of the movement of cilia on oyster gill kept under the absence of oxygen. *Tohoku University Science Reports, Fourth Series, Biology* 28: 85-95.

VALLEE, B.L. & ULMER, D.D. 1972. Biochemical effects of mercury, cadmium and lead. *Annual Review of Biochemistry* 41: 91-128.

VAN HOOK, R.I., BLAYLOCK, B.G., BONDIETTI, E.A., FRANCIS, C.W., HUCKABEE, J.E., REICHLE, D.E., SWEETON, F.H. & WITHERSPOON, J.P. 1974. Radioisotope techniques to evaluate the environmental behaviour of cadmium. In *Comparative Studies of Food and Environmental Contamination*, IAEA-SM-175/22, Vienna.

WALDICHUCK, M. 1973. Trends in methodology for evaluation of effects of pollutants on marine organisms and ecosystems. *CRC Critical Reviews in Environmental Control*, pp. 167-211, CRC Press.

WALDICHUCK, M. 1974. Some biological concerns in heavy metals pollution. In *Pollution and Physiology of Marine Organisms* (edited by VERNBERG, F.J. & VERNBERG, W.B.), Academic Press, New York.

WILLIAMS, C.H. & DAVID, D.J. 1973. The effect of superphosphate on the cadmium content of soils and plants. *Australian Journal of Soil Research* 11: 43-56.

WINDOM, H.L. & SMITH, R.G. 1972. Distribution of iron, magnesium, copper, zinc and silver in oysters along the Georgia coast. *Journal of the Fisheries Research Board of Canada* 29: 450-452.

WINGE, D., KRASNO, J. & COLUCCI, A.V. 1974. Cadmium accumulation in rat liver: correlation between bound metal and pathology. In *Trace Element Metabolism in Animals-2*, (edited by HOESTRA, W.G., SUTTIE, J.W., GANTHER, H.E. & W. MERTZ), University Park Press, Baltimore.

WOLFE, D.A. 1970a. Levels of stable zinc and ^{65}zinc in *Crassostrea virginica* from North Carolina. *Journal of the Fisheries Research Board of Canada* 27: 47-57.

WOLFE, D.A. 1970b. Zinc enzymes in *Crassostrea virginica*. *Journal of the Fisheries Research Board of Canada* 27: 59-69.

WOLFE, D.A. & COBURN, C.B. 1970. Influence of salinity and temperature on the accumulation of cesium-137 by an estuarine clam under laboratory conditions. *Health Physics* 18: 499-505.

WOOD, J.M. 1974. Biological cycles for toxic elements in the environment. *Science* 183: 1049-1052.

YEAGER, D.W., CHOLAK, J. & MEINERS, B.G. 1973. The determination of cadmium in biological and related material by atomic absorption. *American Industrial Hygiene Association Journal* 34(10): 450-454.

YONGE, C.M. 1926. Structure and physiology of the organs of feeding and digestion in *Ostrea edulis*. *Journal of the Marine Biological Association* (U.K.) 14: 295-386.

YOSHIKAWA, H., OHSAWA, M. & KANETA, M. 1974. Clinico-chemical studies on subacute cadmium poisoning in rabbits. *Industrial Health* 12: 127-140.

ZAROOGIAN, G.E. & CHEER, S. 1976. Accumulation of cadmium by the American oyster *Crassostrea virginica*. *Nature* (London) 261: 408-410.

ZIRINO, A. & YAMAMOTO, S. 1972. A pH-dependent model for the chemical speciation of copper, zinc, cadmium and lead in sea water. *Limnology and Oceanography* 17(5) 661-671.

ZS-NAGY, I. & ERMANI, M. 1972. ATP production in the tissue of the bivalve, *Mytilus galloprovincalialis* (Pelecypoda), under normal and anoxic conditions. *Comparative Biochemistry and Physiology* 43B: 593-600.

ZWAAN, A. DE. & MARREWIJK, W.J.A. 1973. Intracellular localization of pyruvate carboxylase, phosphoenolpyruvate carboxylase and malic enzyme, and the absence of glyoxylate cycle enzymes in the sea mussell *Mytilus edulis*. *Comparative Biochemistry and Physiology* 44B: 1057-1066.